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<b>(54) Title:</b> USE OF INTERLEUKIN-10 TO SUPPRESS GRAFT-VS.-HOST DISEASE			
<b>(57) Abstract</b>  A method is provided for suppressing graft-vs.-host disease or tissue rejection which comprises administering to an individual an effective amount of interleukin-10.			

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## USE OF INTERLEUKIN-10 TO SUPPRESS GRAFT-VS.-HOST DISEASE

### Field of the Invention

The invention relates generally to a method for treating and  
5 inhibiting graft-vs.-host disease or tissue rejection by administering to an afflicted individual an effective amount of interleukin-10.

### SUMMARY OF THE INVENTION

The invention relates to the use of interleukin-10 (IL-10) to suppress graft-vs.-host disease or the rejection of transplanted tissues. The  
10 invention also includes pharmaceutical compositions comprising interleukin-10 or active variants thereof. Preferably, the interleukin-10 of the invention is selected from the group consisting of the mature polypeptides having the open reading frames that are defined by the amino acid sequences given in SEQ. ID. NOS. 1 and 2 herein (all SEQ.  
15 IDs. are given immediately before the Claims), wherein the standard three-letter abbreviation is used to indicate L-amino acids, starting from the N-terminus. These two forms of IL-10 are sometimes referred to as human IL-10 (or human cytokine synthesis inhibitory factor) and viral IL-10 (or BCRF1), respectively: e.g. Moore et al., *Science*, Vol. 248, pgs. 1230-1234  
20 (1990); Vieira et al., *Proc. Natl. Acad. Sci.*, Vol. 88, pgs. 1172-1176 (1991); Fiorentino et al., *J. Exp. Med.*, Vol. 170, pgs. 2081-2095 (1989); Hsu et al., *Science*, Vol. 250, pgs. 830-832 (1990). More preferably, the mature IL-10 or variants thereof used in the methods of the invention are selected from the group consisting of the mature polypeptides having the open reading  
25 frames that are defined by the amino acid sequences given in SEQ. ID. NOS. 3 and 4 herein.

### Brief Description of the Drawings

Figure 1 is a diagram of the vector pcD(SR $\alpha$ ) used for expressing IL-10 in mammalian cells.

30 Figure 2 is a diagram of the vector TRP-C11 used for expressing IL-10 in bacteria.

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Figure 3 shows plasmid pGSRG carrying the open reading frame (ORF) of mouse IL-10, viral IL-10, or human IL-10 inserted into its *Xho*I restriction site; it also shows the sequence of the RBS-ATG-polylinker regions of the final construction (called TAC-RBS).

5        Figure 4 shows the effects of endogenous and exogenous IL-10 on the proliferative responses in MLC. PBMC ( $1 \times 10^5$ /well) and allogeneic irradiated PBMC ( $1 \times 10^5$ /well) (PBMC donor A x PBMC donor B (A); PBMC donor B x PBMC donor A (B)) were cultured for 5 days in the presence of increasing concentrations of IL-10 (open bars) and anti-IL-10  
10        mAb (solid bars). MLC were carried out in the absence (solid bars) or in the presence (hatched bars) of 100 U/ml IL-10 and increasing concentrations of anti-IL-10 mAb (C).

Figure 5 shows the effects of IL-10 on the proliferative responses of purified T cells stimulated with various allogeneic cells. Purified T cells  
15        ( $1 \times 10^5$ /well) were cultured for 5 days with allogeneic irradiated elutriated monocytes ( $2 \times 10^4$ /well) (A), positively sorted CD14<sup>+</sup> monocytes ( $2 \times 10^4$ /well) (B); purified B cells ( $3.3 \times 10^4$ /well) (C), EBV-LCL ( $1 \times 10^4$ /well) (D) in the presence of increasing concentrations of IL-10.

Figure 6 shows how the kinetics of the IL-10 effects depend upon  
20        the time that the IL-10 is added to the culture. PBMC ( $1 \times 10^5$ /well) and allogeneic irradiated PBMC ( $1 \times 10^5$ /well) were cultured for 5 days. The indicated concentrations of IL-10 were added at times indicated.

Figure 7 shows the effects of IL-10 on IL-2-production in MLC. PBMC ( $1 \times 10^5$ /well) and allogeneic irradiated PBMC ( $1 \times 10^5$ /well) were  
25        cultured with increasing concentrations of IL-10 and in the presence or in the absence of 10  $\mu$ g/ml of the anti-IL-2 R antibody BB10. Three days later the supernatants were harvested and assayed for their IL-2 content by cytokine-specific ELISA.

Figure 8 shows the effect of exogenous IL-2 on the reduced  
30        alloantigen-induced proliferative response of T cells induced by IL-10. Purified T cells ( $10^5$ /well) stimulated with allogeneic irradiated PBMC ( $10^5$ /well) (A), or purified B cells ( $3.3 \times 10^4$ /well) (B), were cultured with increasing amounts of IL-2 in the absence (open symbols) or in the presence (closed symbols) of 100 U/ml of IL-10.

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### DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to a method of using IL-10 or agonists thereof to suppress graft-vs.-host disease or tissue rejection in individuals, e.g., transplant patients. The invention also includes pharmaceutical compositions comprising IL-10 for carrying out the method. IL-10 for use in the invention is selected from the group of mature polypeptides encoded by the open reading frames defined by the cDNA inserts of pH5C, pH15C, and pBCRF1(SR $\alpha$ ), which are deposited with the American Type Culture Collection (ATCC), Rockville, Maryland, under accession numbers 68191, 68192, and 68193, respectively, and active variants thereof, e.g., agonists. Agonists include both muteins and post-translational variants of, e.g., processing, truncation, glycosylation.

#### I. Assays for Interleukin-10

IL-10s exhibit several biological activities which could form the basis of assays and units. In particular, IL-10s have the property of inhibiting the synthesis of at least one cytokine in the group consisting of IFN- $\gamma$ , lymphotoxin, IL-2, IL-3, and GM-CSF in a population of T helper cells induced to synthesize one or more of these cytokines by exposure to syngeneic antigen-presenting cells (APCs) and antigen. In this activity, the APCs are treated so that they are incapable of replication, but that their antigen-processing machinery remains functional. This is conveniently accomplished by irradiating the APCs, e.g. with about 1500-3000 R (gamma or X-radiation) before mixing with the T cells.

Alternatively, cytokine inhibition may be assayed in primary or, preferably, secondary mixed lymphocyte reactions (MLR), in which case syngeneic APCs need not be used. MLRs are well known in the art, e.g. Bradley, pgs. 162-166, in Mishell et al., eds. Selected Methods in Cellular Immunology (Freeman, San Francisco, 1980); and Battisto et al., *Meth. in Enzymol.*, Vol. 150, pgs. 83-91 (1987). Briefly, two populations of allogeneic lymphoid cells are mixed, one of the populations having been treated prior to mixing to prevent proliferation, e.g. by irradiation. Preferably, the cell populations are prepared at a concentration of about  $2 \times 10^6$  cells/ml in supplemented medium, e.g. RPMI 1640 with 10% fetal calf serum. For both controls and test cultures, mix 0.1 ml of each

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population for the assay. For a secondary MLR, the cells remaining after 7 days in the primary MLR are re-stimulated by freshly prepared, irradiated stimulator cells. The sample suspected of containing IL-10 may be added to the test cultures at the time of mixing, and both controls and test cultures  
5 may be assayed for cytokine production from 1 to 3 days after mixing.

Obtaining T cell populations and/or APC populations for IL-10 assays employs techniques well known in the art which are fully described in DiSabato et al., eds., *Meth. in Enzymol.*, Vol. 108 (1984). APCs for the preferred IL-10 assay are peripheral blood monocytes. These are  
10 obtained using standard techniques, e.g. as described by Boyum, *Meth. in Enzymol.*, Vol. 108, pgs. 88-102 (1984); Mage, *Meth. in Enzymol.*, Vol. 108, pgs. 118-132 (1984); Litvin et al., *Meth. in Enzymol.*, Vol. 108, pgs. 298-302 (1984); Stevenson, *Meth. in Enzymol.*, Vol. 108, pgs. 242-249 (1989); and Romain et al., *Meth. in Enzymol.*, Vol. 108, pgs. 148-153  
15 (1984); all of which references are incorporated herein by reference. Preferably, helper T cells are used in the IL-10 assays, which are obtained by first separating lymphocytes from the peripheral blood and then selecting, e.g. by panning or flow cytometry, helper cells using a commercially available anti-CD4 antibody, e.g. OKT4 described in U.S.  
20 patent 4,381,295 and available from Ortho Pharmaceutical Corp. The requisite techniques are fully disclosed by Boyum in *Scand. J. Clin. Lab. Invest.*, Vol. 21 (Suppl. 97), pg. 77 (1968), and in *Meth. in Enzymol.*, Vol. 108 (cited above), and by Bram et al. in *Meth. in Enzymol.*, Vol. 121, pgs. 737-748 (1986). Generally, PBLs are obtained from fresh blood by Ficoll-  
25 Hypaque density gradient centrifugation.

A variety of antigens can be employed in the assay, e.g. Keyhole limpet hemocyanin (KLH), fowl  $\gamma$ -globulin, or the like. More preferably, in place of antigen, helper T cells are stimulated with anti-CD3 monoclonal antibody, e.g. OKT3 disclosed in U.S. patent 4,361,549, in the assay.

30 Cytokine concentrations in control and test samples are measured by standard biological and/or immunochemical assays. Construction of immunochemical assays for specific cytokines is well known in the art when the purified cytokine is available: e. g. Campbell, *Monoclonal Antibody Technology* (Elsevier, Amsterdam, 1984); Tijssen, *Practice and*  
35 *Theory of Enzyme Immunoassays* (Elsevier, Amsterdam, 1985); and U.S. patent 4,486,530 are exemplary of the extensive literature on the subject.

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ELISA kits for human IL-2, human IL-3, and human GM-CSF are commercially available from Genzyme Corp. (Boston, MA); and an ELISA kit for human IFN- $\gamma$  is commercially available from Endogen, Inc. (Boston, MA). Polyclonal antibodies specific for human lymphotoxin are available  
5 from Genzyme Corp. which can be used in a radioimmunoassay for human lymphotoxin, e.g. Chard, *An Introduction to Radioimmunoassay and Related Techniques* (Elsevier, Amsterdam, 1982).

Biological assays of the cytokines listed above can also be used to determine IL-10 activity. A biological assay for human lymphotoxin is  
10 disclosed by Aggarwal, *Meth. in Enzymol.*, Vol. 116, pgs. 441-447 (1985), and Matthews et al., pgs. 221-225, in Clemens et al., eds., *Lymphokines and Interferons: A Practical Approach* (IRL Press, Washington, D.C., 1987). Human IL-2 and GM-CSF can be assayed with factor dependent cell lines CTLL-2 and KG-1, available from the ATCC under accession numbers TIB  
15 214 and CCL 246, respectively. Human IL-3 can be assayed by its ability to stimulate the formation of a wide range of hematopoietic cell colonies in soft agar cultures, e.g. as described by Metcalf, *The Hemopoietic Colony Stimulating Factors* (Elsevier, Amsterdam, 1984). IFN- $\gamma$  can be quantified with anti-viral assays, e.g. Meager, pgs. 129-147, in Clemens et al., eds.  
20 (cited above). See also, Roitt (1992) Encyclopedia of Immunology, Academic Press, New York; and Coligan (1992 and periodic supplements) Current Protocols in Immunology Greene/Wiley, New York.

Cytokine production can also be determined by mRNA analysis. Cytokine mRNAs can be measured by cytoplasmic dot hybridization as  
25 described by White et al., *J. Biol. Chem.*, Vol. 257, pgs. 8569-8572 (1982), and Gillespie et al., U.S. patent 4,483,920. Accordingly, these references are incorporated by reference. Other approaches include dot blotting using purified RNA, e.g. chapter 6, in Hames et al., eds., *Nucleic Acid Hybridization A Practical Approach* (IRL Press, Washington, D.C., 1985).

30 Some samples to be tested for IL-10 activity may require pretreatment to remove predetermined cytokines that might interfere with the assay. For example, IL-2 increases the production of IFN- $\gamma$  in some cells. Thus depending on the helper T cells used in the assay, IL-2 may have to be removed from the sample being tested. Such removals are  
35 conveniently accomplished by passing the sample over a standard anti-cytokine affinity column.

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- For convenience, units of IL-10 activity are defined in terms of IL-10's ability to augment the IL-4-induced proliferation of MC/9 cells, which are described in U.S. patent 4,559,310 and available from the ATCC under accession number CRL 8306. 1 unit/ml is defined as the
- 5 concentration of IL-10 which gives 50% of maximum stimulation of MC/9 proliferation above the level of IL-4 in the following assay. Prepare duplicate or triplicate dilutions of IL-4 and IL-10 in 50  $\mu$ l of medium per well in a standard microtiter plate. Medium consists of RPMI 1640, 10% fetal calf serum, 50  $\mu$ M 2-mercaptoethanol, 2 mM glutamine, penicillin (100 U/L)
- 10 and streptomycin (100  $\mu$ g/L). Add IL-4, 25  $\mu$ l/well of 1600 U/ml (400 U/ml final) diluted in medium and incubate overnight, e.g. 20-24 hours. Add  $^3$ H-thymidine (e.g. 50  $\mu$ Ci/ml in medium) at 0.5-1.0  $\mu$ Ci/well and again incubate the cells overnight; thereafter harvest the cells and measure the incorporated radioactivity.
- 15 Variants and analogs of the IL-10 described herein can be made by recombinant means as described in, e.g., Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual Cold Spring Harbor Press, Cold Spring Harbor, New York; or Ausubel (1987 and periodic supplements) Current Protocols in Molecular Biology Greene/Wiley, New York; or by
- 20 synthetic techniques, as described, e.g., in Atherton et al. (1989) Solid Phase Peptide Synthesis: A Practical Approach IRL Press, Oxford.

## II. Purification and Pharmaceutical Compositions

- When polypeptides of the present invention are expressed in soluble form, for example as a secreted product of transformed yeast or
- 25 mammalian cells, they can be purified according to standard procedures of the art, including steps of ammonium sulfate precipitation, ion exchange chromatography, gel filtration, electrophoresis, affinity chromatography, and/or the like: e.g. "Enzyme Purification and Related Techniques," *Methods in Enzymology*, 22:233-577 (1977); and Scopes, R., *Protein*
- 30 *Purification: Principles and Practice* (Springer-Verlag, New York, 1982) provide guidance in such purifications. Likewise, when polypeptides of the invention are expressed in insoluble form, for example as aggregates, inclusion bodies, or the like, they can be purified by standard procedures in the art, including separating the inclusion bodies from disrupted host cells



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by centrifugation, solubilizing the inclusion bodies with chaotropic and reducing agents, diluting the solubilized mixture, and lowering the concentration of chaotropic agent and reducing agent so that the polypeptide takes on a biologically active conformation. The latter

5 procedures are disclosed in the following references, which are incorporated by reference: Winkler et al., *Biochemistry*, 25: 4041-4045 (1986); Winkler et al., *Biotechnology*, 3:992-998 (1985); Koths et al., U.S. patent 4,569,790; and European patent applications 86306917.5 and 86306353.3.

10 As used herein "effective amount" means an amount sufficient to reduce or prevent graft-vs.-host disease or tissue rejection. See, e.g., Paul (1989) Fundamental Immunology Raven Press, New York. The effective amount for a particular patient may vary depending on such factors as the state, type, and amount of tissue transplanted, the overall health of the

15 patient, method of administration, the severity of side-effects, and the like. Generally, IL-10 is administered as a pharmaceutical composition comprising an effective amount of IL-10 and a pharmaceutical carrier. A pharmaceutical carrier can be any compatible, non-toxic substance suitable for delivering the compositions of the invention to a patient.

20 Generally, compositions useful for parenteral administration of such drugs are well known, e.g. Remington's *Pharmaceutical Science*, 15th Ed. (Mack Publishing Company, Easton, PA 1980). Alternatively, compositions of the invention may be introduced into a patient's body by implantable or injectable drug delivery system, e.g. Urquhart et al., *Ann. Rev. Pharmacol.*

25 *Toxicol.*, Vol. 24, pgs. 199-236 (1984); Lewis, ed. *Controlled Release of Pesticides and Pharmaceuticals* (Plenum Press, New York, 1981); U.S. patent 3,773,919; U.S. patent 3,270,960; and the like.

When administered parenterally, the IL-10 is formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with

30 a pharmaceutical carrier. Examples of such carriers are normal saline, Ringer's solution, dextrose solution, and Hank's solution. Nonaqueous carriers such as fixed oils and ethyl oleate may also be used. A preferred carrier is 5% dextrose/saline. The carrier may contain minor amounts of additives such as substances that enhance isotonicity and chemical

35 stability, e.g., buffers and preservatives. The IL-10 is preferably formulated in purified form substantially free of aggregates and other proteins at a

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concentration in the range of about 5 to 20  $\mu\text{g/ml}$ . Preferably, IL-10 is administered by continuous infusion so that an amount in the range of about 50-800  $\mu\text{g}$  is delivered per day (i.e. about 1-16  $\mu\text{g/kg/day}$ ). The daily infusion rate may be varied based on monitoring of side effects and blood cell counts.

### EXAMPLES

The following examples serve to illustrate the present invention. The selected vectors and hosts, the concentration of reagents, the temperatures, and the values of other variables are only to exemplify application of the present invention and are not to be considered limitations thereof.

#### Example 1. Expression of human CSIF in a bacterial host

A synthetic human CSIF gene is assembled from a plurality of chemically synthesized double-stranded DNA fragments to form an expression vector designated TAC-RBS-hCSIF. Cloning and expression are carried out in a standard bacterial system, for example *E. coli* K-12 strain JM101, JM103, or the like, described by Viera and Messing, in *Gene*, Vol. 19, pgs. 259-268 (1982). Restriction endonuclease digestions and ligase reactions are performed using standard protocols, e.g. Maniatis et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1982).

The alkaline method (Maniatis et al., cited above) is used for small scale plasmid preparations. For large scale preparations a modification of the alkaline method is used in which an equal volume of isopropanol is used to precipitate nucleic acids from the cleared lysate. Precipitation with cold 2.5 M ammonium acetate is used to remove RNA prior to cesium chloride equilibrium density centrifugation and detection with ethidium bromide.

For filter hybridizations Whatman 540 filter circles are used to lift colonies which are then lysed and fixed by successive treatments with 0.5M NaOH, 1.5M NaCl; 1M Tris.HCl pH8.0, 1.5M NaCl (2 min each); and heating at 80°C for 2 hours. Hybridizations are in 6xSSPE, 50% formamide, 0.1% sodium dodecylsulphate (SDS), 100  $\mu\text{g/ml}$  *E. coli* tRNA

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- at 42°C for 6 hours using <sup>32</sup>P-labelled (kinased) synthetic DNAs. (20xSSPE is prepared by dissolving 174 g of NaCl, 27.6 g of NaH<sub>2</sub>PO<sub>4</sub>·9H<sub>2</sub>O, and 7.4 g of EDTA in 800 ml of H<sub>2</sub>O. pH is adjusted to 7.4 with NaOH, volume is adjusted to 1 liter, and the whole is sterilized by autoclaving.) Filters are washed twice (15 min, room temperature) with 1xSSPE, 0.1% SDS. After autoradiography (Fuji RX film), positive colonies are located by aligning the regrown colonies with the blue-stained colonies on the filters. DNA is sequenced by the dideoxy method of Sanger et al. *Proc. Natl. Acad. Sci.*, Vol. 74, pg. 5463 (1977). Templates for the dideoxy reactions are either single-stranded DNAs of relevant regions recloned into M13mp vectors, e.g. Messing et al. *Nucleic Acids Res.*, Vol. 9, pg. 309 (1981); or double-stranded DNA prepared by the minialkaline method and denatured with 0.2M NaOH (5 min, room temperature) and precipitated from 0.2M NaOH, 1.43M ammonium acetate by the addition of 2 volumes of ethanol. DNA is synthesized by phosphoramidite chemistry using Applied Biosystems 380A synthesizers. Synthesis, deprotection, cleavage and purification (7M urea PAGE, elution, DEAE-cellulose chromatography) are done as described in the 380A synthesizer manual.
- Complementary strands of synthetic DNAs to be cloned (400 ng each) are mixed and phosphorylated with polynucleotide kinase in a reaction volume of 50 µl. This DNA is ligated with 1 µg of vector DNA digested with appropriate restriction enzymes, and ligations are in a volume of 50 µl at room temperature for 4 to 12 hours. Conditions for phosphorylation, restriction enzyme digestions, polymerase reactions, and ligation have been described (Maniatis et al., cited above). Colonies are scored for lacZ<sup>+</sup> (when desired) by plating on L agar supplemented with ampicillin, isopropyl-1-thio-beta-D-galactoside (IPTG) (0.4 mM) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (x-gal) (40 mg/ml).
- The TAC-RBS vector is constructed by filling-in with DNA polymerase the single *Bam* HI site of the tacP-bearing plasmid pDR540 (Pharmacia). This is then ligated to unphosphorylated synthetic oligonucleotides (Pharmacia) which form a double-stranded fragment encoding a consensus ribosome binding site as given in SEQ. ID. NO. 5 herein and designated RBS. After ligation, the mixture is phosphorylated and religated with the *Sst*I linker ATGAGCTCAT. This complex is then cleaved

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with *Sst*I and *Eco*RI, and the 173 base pair (bp) fragment isolated by polyacrylamide gel electrophoresis (PAGE) and cloned into *Eco*RI-*Sst*I-restricted pUC19 (Pharmacia) (as described below). The sequence of the RBS-ATG-polylinker regions of the final construction (called TAC-RBS) is shown in Figure 2.

The synthetic IL-10 gene is assembled into a pUC19 plasmid in eight steps. At each step inserts free of deletions and/or inserts can be detected after cloning by maintaining the *lacZ*( $\alpha$ ) gene of pUC19 in frame with the ATG start codon inserted in step 1. Clones containing deletion and/or insertion changes can be filtered out by scoring for blue colonies on L-ampicillin plates containing x-gal and IPTG. Alternatively, at each step sequences of inserts can be readily confirmed using a universal sequencing primer on small scale plasmid DNA preparations, e.g. available from Boehringer Mannheim.

In step 1, the TAC-RBS vector is digested with *Sst*I, treated with T4 DNA polymerase (whose 3'-exonuclease activity digests the 3'-protruding strands of the *Sst*I cuts to form blunt-end fragments), and after deactivation of T4 DNA polymerase, treated with *Eco*RI to form a 173 bp fragment containing the TAC-RBS region and having a blunt end at the ATG start codon and the *Eco*RI cut at the opposite end. Finally, the 173 bp TAC-RBS fragment is isolated.

In step 2, the isolated TAC-RBS fragment of step 1 is mixed with *Eco*RI/*Kpn*I-digested plasmid pUC19 and synthetic fragment 1A/B whose nucleic acid sequences are shown in SEQ. ID. NOs. 6 and 7 herein, which has a blunt end at its upstream terminus and a staggered end corresponding to a *Kpn*I cut at its downstream terminus. This *Kpn*I end is adjacent to and downstream of a *Bst*EII site. The fragments are ligated to form the pUC19 of step 2.

In step 3, synthetic fragments 2A/B and 3A/B are mixed with *Bst*EII/*Sma*I-digested pUC19 of step 2 (after amplification and purification) and ligated to form pUC19 of step 3. The nucleic acid sequences of synthetic fragment 2A/B are shown in SEQ. ID. NOs. 8 and 9 herein and the nucleic acid sequences of synthetic fragment 3A/B are shown in SEQ. ID. NOs. 10 and 11 herein. Note that the downstream terminus of fragment 3A/B contains extra bases which form the *Sma*I blunt-end. These extra bases are cleaved in step 4. Also, fragments 2A/B

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and 3A/B have complementary 9-residue single-stranded ends which anneal upon admixture, leaving the upstream *Bst* EII cut of 2A/B and the downstream blunt end of 3A/B to ligate to the pUC19.

5 In step 4, the pUC19 of step 3 is digested with *Afl*II/*Xba*I, amplified, purified, repurified, mixed with synthetic fragment 4A/B whose nucleic acid sequences are shown in SEQ. ID. NOs. 12 and 13 herein, and ligated to form pUC19 of step 4.

10 In step 5, the pUC19 of step 4 is digested with *Xba*I/*Sa*I, amplified and purified, and mixed with synthetic fragment 5A/B whose nucleic acid sequences are shown in SEQ. ID. NOs. 14 and 15 herein and ligated to form the pUC19 of step 5. Note that the *Sa*I-staggered end of fragment 5A/B is eliminated by digestion with *Hpa*I in step 6.

15 In step 6, the pUC19 of step 5 is digested with *Hpa*I/*Pst*I, amplified and purified, and mixed with synthetic fragment 6A/B whose nucleic acid sequences are shown in SEQ. ID. NOs. 16 and 17 herein and ligated to form the pUC19 of step 6.

20 In step 7, the pUC19 of step 6 is digested with *Cla*I/*Sph*I, amplified and purified, and mixed with synthetic fragment 7A/B whose nucleic acid sequences are shown in SEQ. ID. NOs. 18 and 19 herein and ligated to form the pUC19 of step 7.

25 In step 8, the pUC19 of step 7 is digested with *Mlu*I/*Hin*dIII, amplified and purified, and mixed with synthetic fragments 8A/B and 9A/B and ligated to form the final construction, which is then inserted into *E. coli* K-12 strain JM101, e.g. available from the ATCC under accession number 33876, by standard techniques. The nucleic acid sequences of synthetic fragment 8A/B are shown in SEQ. ID. NOs. 20 and 21 herein and the nucleic acid sequences of synthetic fragment 9A/B are shown in SEQ. ID. NOs. 22 and 23 herein. After cultivation, protein is extracted from the JM101 cells and dilutions of the extracts are tested for biological activity.

30 Example 2. Expression of vIL-10 in COS 7 Monkey cells

A gene encoding the open reading frame of vIL-10 was amplified by polymerase chain reaction using primers that allowed later insertion of the amplified fragment into an *Eco*RI-digested pcD(SR $\alpha$ ) vector (Figure 1).

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The coding strand of the inserted fragment is shown in SEQ. ID. NO. 15 herein.

Clones carrying the insert in the proper orientation were identified by expression of vIL-10 and/or the electrophoretic pattern of restriction digests. One such vector carrying the vIL-10 gene was designated pBCRF1(SR $\alpha$ ) and was deposited with the ATCC under accession number 68193. pBCRF1(SR $\alpha$ ) was amplified in *E. coli* MC1061, isolated by standard techniques, and used to transfect COS 7 monkey cells as follows: One day prior to transfection, approximately  $1.5 \times 10^6$  COS 7 monkey cells were seeded onto individual 100 mm plates in Dulbecco's modified Eagle medium (DME) containing 5% fetal calf serum (FCS) and 2 mM glutamine. To perform the transfection, COS 7 cells were removed from the dishes by incubation with trypsin, washed twice in serum-free DME, and suspended to  $10^7$  cells/ml in serum-free DME. A 0.75 ml aliquot was mixed with 20  $\mu$ g DNA and transferred to a sterile 0.4 cm electroporation cuvette. After 10 minutes, the cells were pulsed at 200 volts, 960  $\mu$ F in a BioRad Gene Pulser unit. After another 10 minutes, the cells were removed from the cuvette and added to 20 ml of DME containing 5% FCS, 2mM glutamine, penicillin, streptomycin, and gentamycin. The mixture was aliquoted to four 100 mm tissue culture dishes. After 12-24 hours at 37°C, 5% CO<sub>2</sub>, the medium was replaced with similar medium containing only 1% FCS and the incubation continued for an additional 72 hours at 37°C, 5% CO<sub>2</sub>, after which the medium was collected and assayed for its ability to inhibit IFN- $\gamma$  synthesis.

10 ml aliquots of freshly isolated PBLs (about  $2 \times 10^6$  cells/ml) were incubated at 37°C with PHA (100 ng/ml) in medium consisting of (i) 90% DME supplemented with 5% FCS and 2 mM glutamine, and (ii) 10% supernatant from COS 7 cells previously transfected with pBCRF1(SR $\alpha$ ). After 24 hours the cells and supernatants were harvested to assay for the presence of either IFN- $\gamma$  mRNA or IFN- $\gamma$  protein, respectively. Controls were treated identically, except that the 10% supernatant was from COS 7 cultures previously transfected with a plasmid carrying an unrelated cDNA insert. The vIL-10-treated samples exhibited about a 50% inhibition of IFN- $\gamma$  synthesis relative to the controls.

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Example 3. Expression of vIL-10 in Escherichia coli

A gene encoding the mature vIL-10 shown in SEQ. ID. NO. 4 herein may be expressed in *E. coli*.

5 The cDNA insert of pBCRF1(SR $\alpha$ ) is recloned into an M13 plasmid where it is altered twice by site-directed mutagenesis: first to form a *Cla*I site at the 5'-end of the coding region for the mature vIL-10 polypeptide, and second to form a *Bam*HI site at the 3'-end of the coding region for the mature vIL-10 polypeptide. The mutated sequence is then readily inserted into the TRPC11 expression vector described below.

10 The TRPC11 vector was constructed by ligating a synthetic consensus RBS fragment to *Cla*I linkers (ATGCAT) and by cloning the resulting fragments into *Cla*I-restricted pMT11hc (which had been previously modified to contain the *Cla*I site). pMT11hc is a small (2.3 kilobase) high copy, AMP<sup>R</sup>, TET<sup>S</sup> derivative of pBR322 that bears the  $\pi$ VX plasmid *Eco*RI-*Hin*dIII polylinker region. ( $\pi$ VX is described by Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). This was modified to contain the *Cla*I site by restricting pMT11hc with *Eco*RI and *Bam*HI, filling in the resulting sticky ends and ligating with *Cla*I linker (CATCGATG), thereby restoring the *Eco*RI and  
20 *Bam*HI sites and replacing the *Sma*I site with a *Cla*I site. One transformant from the TRPC11 construction had a tandem RBS sequence flanked by *Cla*I sites. One of the *Cla*I sites and part of the second copy of the RBS sequence were removed by digesting this plasmid with *Pst*I, treating with Bal31 nuclease, restricting with *Eco*RI and treating with T4  
25 DNA polymerase in the presence of all four deoxynucleotide triphosphates. The resulting 30-40 bp fragments were recovered by PAGE and cloned into *Sma*I-restricted pUC12. A 248 bp *E. coli* trpP-bearing *Eco*RI fragment derived from pKC101 (described by Nichols et al. in *Methods in Enzymology*, Vol. 101, pg. 155 (Academic Press, N.Y. 1983)) was then  
30 cloned into the *Eco*RI site to complete the TRPC11 construction, which is illustrated in Figure 2. TRPC11 is employed as a vector for vIL-10 by first digesting it with *Cla*I and *Bam*HI, purifying it, and then mixing it in a standard ligation solution with the *Cla*I-*Bam*HI fragment of the M13 containing the nucleotide sequence coding for the mature BCRF1. The  
35 insert-containing TRPC11, referred to as TRPC11-BCRF1, is propagated in

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*E. coli* K12 strain JM101, e.g. available from the ATCC under accession number 33876.

#### Example 4

5 This Example was disclosed after the priority date of this document by Bejarano et al. (1992) International Immunology 4:1389-1397. It provides *in vitro* evidence for the effectiveness of IL-10 treatment in appropriate circumstances. More particularly, it demonstrates that IL-10 inhibits allogeneic proliferative and cytotoxic T cell responses generated in primary mixed lymphocyte cultures (MLC).

10 This Example shows how IL-10 inhibited the alloantigen-induced proliferative responses in a dose-dependent fashion. The suppressive effect was optimal when IL-10 was added at the beginning of the cultures, suggesting that it acts on the early stages of T cell activation. The proliferative responses were enhanced in the presence of anti-IL-10 mAb, 15 indicating that endogenously produced IL-10 suppresses proliferation in primary MLC. Whether the stimulator cells were irradiated allogeneic peripheral blood mononuclear cells (PBMC), purified monocytes, or B cells, the inhibitory effects of IL-10 were still observed. The reduced proliferative responses were not restored by high concentrations of 20 exogenous IL-2, indicating that the effects of IL-10 are not only related to inhibition of IL-2 synthesis. Furthermore, the production of IL-2, IFN- $\gamma$ , IL-6, GM-CSF, and TNF- $\alpha$  in primary MLC was diminished by IL-10 and enhanced in the presence of anti-IL-10 mAb. The strongest effects were observed on the production of IFN- $\gamma$ . Although IL-10 reduces the prolifer- 25 ative responses, the ratio of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells remained the same in IL-10-treated cultures and in control cultures. However, the percentages of activated CD3<sup>+</sup> T cells as judged by CD25<sup>+</sup> and HLA-DR<sup>+</sup> expression were consistently reduced in the presence of IL-10.

30 IL-10 inhibits allospecific proliferative responses and cytokine production. In addition, it was demonstrated that the reduced proliferative responses could not be restored by exogenous IL-12, suggesting that IL-10 inhibits allospecific proliferative T cell responses predominantly by reducing the stimulatory capacity of the stimulator cells.



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These data indicate that IL-10 has important regulatory effects on allogeneic responses *in vitro*.

#### Medium and Reagents.

Cells were cultured in Yssel's medium supplemented with 10%  
5 pooled heat-inactivated human AB serum. See Yssel et al. (1986) Eur. J. Immunol. 16:1187-\_\_\_\_\_.

The neutralizing anti-IL-10 mAb 19F1 was raised against vIL-10 and efficiently neutralized hIL-10 and vIL-10. See Bejarano et al. (1985) Int. J. Cancer 35:327; and ATCC deposit HB10487, deposited June 28, 1990.  
10 The BB10 mAb, which recognizes the IL-2R p55 chain, was a kind gift of Dr. J. Wijdenes (CRTS, Besançon, France; see Herve et al. (19\_\_) Blood 75:1017-1023). Murine anti-CD3 (anti-Leu-4, IgG1), anti-CD4 (anti-Leu-3a, IgG1), anti-CD8 (anti-Leu-2a, IgG2a), anti-CD14 (anti-Leu-M3, IgG2b), anti-CD19 (anti-Leu-12, IgG1), anti-CD25 (anti-IL2R p55, IgG1), anti-CD56  
15 (anti-Leu-19, IgG1), anti-HLA-DR (clone L243, IgG2a) mAb and control mAb of appropriate isotypes were purchased from Becton-Dickinson (Mountain View, CA).

#### Cell Preparations.

Buffy coat preparations were obtained from the Blood Bank of  
20 Stanford University Hospital. PBMC were isolated by density gradient centrifugation over Ficollhpaque (Pharmacia, Uppsala, Sweden).

For purification of T cells, PBMC were depleted of monocytes by plastic adherence and iron phagocytosis. See Bejarano et al. (1985) Int. J. Cancer 35:327-\_\_\_\_\_. Non-adherent cells were passed through nylon wool  
25 (Julius et al. (1973) Eur. J. Immunol. 3:645-\_\_\_\_), and then NK cells were removed by depletion with magnetic beads. Briefly, following staining with saturating concentrations of anti-CD56 mAb for 30 min at 4°C, cells were washed twice with Hanks's balanced salt solution (HBSS), and subsequently rosetted with magnetic beads coated with sheep anti-mouse  
30 IgG (Dynbeads M-450 sheep anti-mouse IgG, Dynal AS, Oslo, Norway) at a bead:cell ratio of 40:1. The mixture was incubated for 30 min at 4°C under gentle shaking before removal of rosetted cells with the magnetic particle concentrator according to the manufacturer's recommendations.

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The resulting cell preparations were >99% CD3+, <1% CD14+, <1% CD19+, <1% CD56+.

For isolation of CD14+ monocytes, PBMC were stained with PE-conjugated CD14 mAb (Becton-Dickinson, Mountain View, CA), washed twice in HBSS and thereafter sorted into CD14+ and CD14- populations using a FACStar-Plus (Becton-Dickinson, Sunnyvale, CA). Reanalysis of the sorted populations showed that more than 99.5% of the purified cells were CD14+. In some experiments monocytes were isolated from peripheral blood by density centrifugation in a blood component separator, followed by centrifugal elutriation (see Figdor et al. (1984) J. Immunol. Methods 68:68-\_\_\_). These monocyte preparations were >95% pure, as judged by nonspecific esterase staining.

Purified B lymphocytes were obtained by magnetic-bead depletion. Briefly, non-adherent PBMC were incubated with saturating concentrations of anti-CD3, anti-CD4, anti-CD8, anti-CD14 and anti-CD56 mAbs for 30 min at 4°C. The cells were washed twice in HBSS and thereafter rosetted with magnetic beads coated with sheep anti-mouse IgG (Dynal AS, Oslo, Norway) at a 40:1 bead:cell ratio. Subsequently, the rosetted cells were depleted as described above. The resulting population consisted of >98% CD19+ cells.

#### Proliferation Assay.

PBMC or highly purified T cells ( $1 \times 10^5$  cells/well), were stimulated by various irradiated (4000 rad) allogeneic stimulator cells. PBMC, CD14+ monocytes, monocytes separated by centrifugal elutriation, and purified B lymphocytes, were used as stimulator cells at R:S ratios of 1:1, 5:1, 5:1, and 3:1, respectively. Cultures were carried out in triplicate in 96-well flat-bottomed microtiter plates in the absence (solid bars; Figures 5-7) or in the presence (hatched bars) of IL-10 in 200µl medium.

Cultures were pulsed with [ $^3\text{H}$ ]TdR during the last 10 hours of a 5-day incubation period and harvested onto fiberglass filters, and the radioactivity was determined by liquid scintillation counting. The results are expressed in Fig. 4 as c.p.m of [ $^3\text{H}$ ]TdR incorporation and represent the means of triplicate cultures.

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### Bulk Cultures.

PBMC or highly purified T cells were cultured with irradiated allogeneic cells at the R:S ratios described above in 50 ml flasks at a concentration of  $1 \times 10^6$  responder cells/ml in the presence or in the  
5 absence of 100 U/ml of IL-10. Five to six days later the supernatants were collected and frozen at  $-20^{\circ}\text{C}$  for determination of their cytokine contents, whereas the cells were recovered for phenotype analysis.

### Fluorescence Analysis

Cells ( $10^5$ ) recovered from the bulk cultures were incubated in  
10 V-bottomed microtiter plates (Flow Laboratories, McLean, Va) with 10  $\mu\text{l}$  of purified PE-conjugated mAb for 30 min at  $4^{\circ}\text{C}$ . In the double-labeling experiments, the cells were washed twice in 1% normal mouse serum after the FITC labeling, and a PE-conjugated mAb was added. The cells were washed twice with HBSS containing 1% BSA and 0.02M  $\text{NaN}_3$  and  
15 thereafter analyzed on a FACScan.

### Lymphokine Determinations.

Supernatants collected from bulk cultures at day 5 or 6 were assayed for the content of GM-CSF, IFN- $\gamma$ , TNF- $\alpha$ , IL-2, IL-4, IL-5, and IL-6 by lymphokine-specific ELISA (Bacchetta et al. (1989) J. Immunol.  
20 144:902-\_\_\_\_). For the quantification of IL-2 production, cultures were carried out in the presence of 10mg/ml of the anti-IL-2 receptor antibody BB10, in order to minimize IL-2 consumption. Supernatants were harvested after 72 hours and the IL-2 levels were determined by specific ELISA. The sensitivity of the various ELISA were: 40 pg/ml for IL-4; 20  
25 pg/ml for IL-2, IL-5 and IL-6; 50 pg/ml for GM-CSF; and 100 pg/ml for TNF- $\alpha$  and IFN- $\gamma$ .

### IL-10 inhibits proliferative responses in MLC.

To determine the effects of IL-10 on the proliferative responses in classical one-way primary MLC, PBMC were stimulated with allogeneic  
30 PBMC in the absence or presence of various concentrations of IL-10. Figure 4 shows that IL-10 inhibited the proliferative responses in a dose-dependent fashion. Significant inhibitory effects were already observed at IL-10 concentrations as low as 1 U/ml, whereas maximal inhibitory effects (ranging from 33 to 95% inhibition in different experiments) were obtained

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at IL-10 concentrations of 100 U/ml. These inhibitory effects of IL-10 were completely neutralized by the anti-IL-10 mAb indicating the specificity of the inhibition (Figure 4c). In fact, the proliferative responses in MLC carried out in the presence of the neutralizing anti-IL-10 mAb were  
5 significantly enhanced, indicating that endogenously produced IL-10 is responsible for suppressing proliferative responses in primary MLC.

Effect of monocytes on the inhibitory effects of IL-10 in MLC.

It is known that IL-10 strongly reduces the Ag-presenting (AP) capacity of monocytes through down-regulation of class II MHC antigens.  
10 In contrast, class II MHC expression and AP-capacity of Epstein-Barr Virus (EBV)-transformed B cells (EBV-transformed lymphoblastoid cell line; EBV-LCL) are not affected by IL-10.

In this experiment, highly enriched T cells obtained by negative selection were used as responder cells. Purified monocyte populations  
15 enriched either by centrifugal elutriation or by direct sorting of CD14<sup>+</sup> cells from PBMC, purified B lymphocytes, and EBV-LCL were used as stimulator cells. IL-10 strongly inhibited the proliferative responses induced by allogeneic monocytes independently of whether the monocytes were obtained by centrifugal elutriation (Figure 5a) or were positively sorted by  
20 the FACS (Figure 5b); whereas the proliferative responses towards allogeneic EBV-LCL remained unaffected (Figure 5d). As observed with specific proliferative responses to soluble antigens, these results indicate that allospecific proliferation is blocked when allogeneic monocytes, but not when allogeneic EBV-LCL, are used as stimulator cells. The  
25 proliferative responses induced by freshly isolated highly purified allogeneic B cells were also inhibited by IL-10 (Figure 5c), indicating that the suppressive effect of IL-10 is also present when B cells are used as stimulators, despite the fact that IL-10 has no measurable effect on class I or class II MHC expression on these cells.

30 Kinetic experiments revealed that the effect of IL-10 on MLC-induced proliferation decreased gradually with time. IL-10 was most effective when added at the beginning of the primary cultures; if added at day 2 or 3 after the onset of the cultures, the effects were only marginal and no clear dose-response effects were observed (Figure 6). These results  
35 indicated that IL-10 acts on the early stages of activation of T cells in MLC.

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IL-10 prevents cytokine production in MLC.

IL-10 has been shown to reduce IFN- $\gamma$  and GM-CSF production by PBMC activated by anti-CD3 or PHA. In addition, IL-10 inhibits the production of cytokines by monocytes. To determine the effect of IL-10 on cytokine production in one-way MLC, allogeneic PBMC were used as responder and as stimulator cells. The cultures were carried out in the absence or in the presence of IL-10 or anti-IL-10 mAb, and supernatants were collected at day 5 and assayed for their cytokine content. Table 1 shows that IFN- $\gamma$ , IL-6, GM-CSF, and TNF- $\alpha$  were produced in MLC, and that IL-10 inhibited the production of these cytokines to various extents. No significant IL-4 production was detected, and the levels of IL-5 were below 100 pg/ml. The production of IL-10 ranged from 1000 to 3000 pg/ml in different experiments. The strongest inhibitory effects of exogenous IL-10 were observed on the production of IFN- $\gamma$ , whereas the weakest inhibitory effects were observed on IL-6 production.

Increased IFN- $\gamma$ , GM-CSF, and TNF- $\alpha$  levels were observed in supernatants of MLC carried out in the presence of anti-IL-10 mAb (Table 1). These enhancing effects of anti-IL-10 mAb on cytokine production were dose-dependent. Taken together, these results indicate that both endogenous and exogenous IL-10 reduce the production of the cytokines tested. To evaluate the effect of IL-10 on IL-2 production in MLC, and to minimize IL-2 consumption by activated T cells, the cultures were carried out in the presence or in the absence of IL-10 and of the anti-IL-2 receptor mAb BB10. In these experiments, IL-10 prevented IL-2 production in a dose-dependent manner (Figure 7). No measurable levels of IL-2 could be detected when IL-10 was added at 100 U/ml.

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TABLE 1.

**EFFECT OF EXOGENOUS AND ENDOGENOUS IL-10 ON  
CYTOKINE PRODUCTION BY ALLOANTIGEN-STIMULATED  
LYMPHOCYTES**

5

	Condition		Cytokine				
	IL-10 (U/ml)	$\alpha$ IL-10 ( $\mu$ g/ml)	IL-6 (ng/ml)	IL-10 (ng/ml)	GM-CSF (pg/ml)	TNF- $\alpha$ (pg/ml)	IFN- $\gamma$ (ng/ml)
A	0		22.2	572	109	79	<1
	1		20.5		41	61	<1
	10		13.0		11	38	<1
	100		12.1		16	47	<1
		0.05	22.9		144	144	<1
		0.5	25.8		165	111	<1
		5	24.9		172	51	<1
A + B	0		35.4	1473	1744	141	29.5
	1		35.3		928	151	20.8
	10		23.9		784	82	18.1
	100		24.7		612	66	11.4
		0.05	36.9		2372	137	33.1
		0.5	39.1		2355	137	45.3
		5	39.1		3487	250	43.8

Human PBMC (A) were cultured for 5 days alone or with allogeneic irradiated PBMC (B) in the absence and in the presence of IL-10 or the anti IL-10 mAb 19F1. Production of cytokines was determined in the supernatants by cytokine-specific ELISAs.

10 Table 1 presents the results of one Experiment out of four.

The inhibitory effects of IL-10 cannot be restored by exogenous IL-2.

To investigate whether the inhibitory effects of IL-10 were observed in the presence of exogenous IL-2, MLC were carried out with various concentrations of IL-2. Figure 8 shows that, when increasing amounts of  
15 IL-2 were added to MLC in which purified T cells were used as responders and PBMC or purified B cells as stimulators, the proliferation was

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- enhanced both in the absence and in the presence of IL-10. However, the inhibitory effects of IL-10 were still present when IL-2 was added at concentrations up to 100 U/ml; and it should be noted that 10 U/ml are sufficient to saturate high affinity IL-2R. Similarly, addition of 400 U/ml of IL-4, which has T cell growth factor activity, failed to restore the reduced proliferative responses induced by IL-10. Taken together these results indicate that the lack of IL-2 is not the limiting factor responsible for the reduced proliferative responses observed when MLC are carried out in the presence of IL-10.
- 10 IL-10 decreases the proportion of activated T cells in MLC.
- In order to determine whether the reduced proliferative responses in MLC in the presence of IL-10 differentially affected CD4<sup>+</sup> or CD8<sup>+</sup> T cell subsets, the proportions of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> cells were determined. In Table 2 it is shown that the total T cell number decreased by 30 to 60% when the T cells were stimulated with allogeneic PBMC, purified monocytes, or B cells in the presence of IL-10. However, the proportion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells remained the same, indicating that IL-10 has no preferential effect on each of these T cells subsets.
- In contrast, the proportion of activated T cells expressing CD25 and HLA-DR antigens was consistently reduced in the IL-10-containing cultures. The strongest reduction was usually observed in MLC where purified CD3<sup>+</sup> T cells were stimulated with purified B cells or monocytes.

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TABLE 2.  
EFFECT OF IL-10 ON ALLOANTIGEN-STIMULATED T CELLS

	T cell count ( $\times 10^{-6}/\text{ml}$ )		% positive cells							
			CD3+CD4+		CD3+CD8+		CD3+CD25+		CD3+DR+	
	— <sup>a</sup>	IL-10	—	IL-10	—	IL-10	—	IL-10	—	IL-10
<u>EXPT. 1</u>										
T + PBMC	1.3	0.76	69	73	21	24	4	2	3	2
T + B cells	1.2	0.72	55	62	28	28	24	18	19	8
<u>EXPT. 2</u>										
T + PBMC	1.4	1.0	ND <sup>b)</sup>	ND	ND	ND	39	27	36	32
T + B cells	0.90	0.68	ND	ND	ND	ND	20	8	18	4
T + mono- cytes <sup>c)</sup>	1.2	0.40	ND	ND	ND	ND	52	24	43	25
<u>EXPT. 3</u>										
T + PBMC	1.02	0.37	77	78	18	16	16	13	15	9
T + mono- cytes <sup>d)</sup>	0.50	0.17	76	79	19	16	7	4	8	5

a) Indicates absence of IL-10

5 b) ND = Not Done

c) Negatively sorted monocytes

d) Positively sorted monocytes (CD14+).

Purified T cells were cultured with allogeneic irradiated PBMC, purified B cells or monocytes in the absence or presence of IL-10 (100 U/ml). Six days later the recovered T cells were counted and phenotyped by indirect immunofluorescence.

The present results show that IL-10 reduced in a dose-dependent fashion the proliferation of alloresponsive T cells in classical one-way primary MLC in which allogeneic PBMC of two different donors were used as responder and irradiated stimulator cells, respectively. These inhibitory effects were completely neutralized by an anti-IL-10 mAb, demonstrating



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the specificity of the inhibition. In addition, it was shown that the proliferative responses were considerably enhanced in the presence of the anti-IL-10 mAb, indicating that endogenous IL-10 production is responsible for suppression of proliferative responses in MLC.

5 IL-10 also reduced the proliferative responses in MLC where highly purified T cells were used as responders and purified monocytes as stimulators. Interestingly, IL-10 was ineffective when purified T cells were stimulated by irradiated allogeneic EBV-LCL. See Fig 5b. These data are consistent with previous findings that IL-10 strongly blocked the specific  
10 proliferative responses of T cells or T cell clones towards soluble antigens or antigenic peptides when monocytes, but not when EBV-LCL, were used as antigen presenting cells (APC). This reduced antigen-presenting capacity was found to be associated with the down-regulatory effect of IL-10 on class II MHC expression on monocytes. In contrast, IL-10 did not  
15 affect class II MHC expression on EBV-LCL. From these data it was concluded that the reduced antigen-specific proliferative T cell responses reflected prevention of activation of the responder cells, rather than a direct suppressive effect on T cell proliferation. This conclusion was further supported by the reduced  $\text{Ca}^{2+}$  fluxes in the responder T cell clones  
20 activated in the presence of IL-10.

MLC-induced proliferation was inhibited by IL-10 not only when monocytes were used as stimulators, but also when purified B cells were used. Several studies have shown that T cell recognition of MHC  
25 alloantigens is mechanistically similar to recognition of viral, bacterial, or other foreign protein antigens. Recently, it has been shown that a significant proportion of MHC class II alloreactive T cell clones recognize processed determinants from human serum proteins in association with allogeneic class II molecules. In contrast to the situation where new MHC-peptide complexes have to be formed to activate antigen-specific T cell  
30 clones, there is no evidence to indicate that new allo-MHC-peptide complexes must be formed on monocytes and B cells to stimulate T cells in a MLC. Moreover, IL-10 does not affect class II MHC membrane expression on human B cells. Therefore, it is unlikely that the inhibitory effects of IL-10 on MLC-induced T cell proliferation can be solely attributed  
35 to a down-regulation of MHC class II expression on the monocytes. It is

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possible that other mechanisms, yet to be defined, are responsible for the reduced stimulatory capacity of B cells in the presence of IL-10.

It has been demonstrated that, in addition to cross-linking of the TCR/CD3 complex by specific alloantigen, LFA-1 – ICAM-1 interactions are required for cytokine production by allospecific T cells. Furthermore, CD28 - B7/BB1 interactions have been shown to be necessary for induction of alloantigen-specific activation of resting T cells, resulting in cytokine production, proliferation and cytotoxic activity. B7 is weakly expressed on resting B cells and monocytes, but is elevated following activation of these cells. However, it could be ruled out that the reduced proliferative and cytotoxic alloresponses were due to down-regulatory effects of IL-10 on the expression of either TCR/CD3 or these accessory molecules. IL-10 did not affect TCR/CD3, LFA-1 expression on the responder T cells, or ICAM-1 and B7 expression on B cells or monocytes used as stimulators.

The data reported here indicate, moreover, that the reduced proliferative responses towards alloantigens also reflect prevention of activation of the responder T cells. IL-10 had to be present from the onset of the cultures to exert its maximal inhibitory effects. In addition, the proportion of activated T cells, as judged by the expression of CD25 and HLA DR antigens, was considerably lower in the IL-10-containing cultures than in the control MLC. Although the total number of CD3<sup>+</sup> T cells generated in MLC carried out in the presence of IL-10 was reduced, IL-10 did not preferentially affect the responses of CD4<sup>+</sup> or CD8<sup>+</sup> T cells, since the proportions of these T cell subsets were comparable to those in control MLC carried out in the absence of IL-10. The reduced expression of CD25 indicates that the inhibitory effect on T cell proliferation in a MLC is not a mere consequence of the cytokine inhibitory activity of IL-10; and this is supported by the finding that IL-10 also reduces the proliferative responses when exogenous IL-2 is added at concentrations that are sufficient to saturate high affinity IL-2 receptors (Figure 8). Collectively, these data suggest that IL-10 reduces the stimulatory capacity of PBMC, monocytes and normal B cells in MLC. The possibility that IL-10 also has direct effects on the T cells cannot be completely excluded. However, the fact that MLC generated with EBV-LCLs as stimulators are not inhibited by IL-10 argues

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against this notion, unless the EBV-LCL can override the inhibitory effect of IL-10.

The levels of cytokines produced in MLC in which total allogeneic PBMC were used as responder and stimulator were also significantly  
5 reduced in the presence of exogenous IL-10. The amounts of IL-2, IFN- $\gamma$ , TNF- $\alpha$ , and GM-CSF were about two- to three-fold lower than those of control MLC carried out in the absence of IL-10. IL-6 production was much less affected; this may be due to the fact that monocytes present in these  
10 cultures already produce considerable amounts of this cytokine very early after activation, before the suppressor activity of IL-10 becomes effective.

The present results therefore clearly indicate that IL-10 plays an important role in down-regulating alloresponsiveness *in vitro*. From these  
*in vitro* observations, and considering that alloantigens are the major targets for specific immunological rejection of transplanted tissue, one can  
15 expect IL-10 to play a role in the induction or maintenance of tolerance following allogeneic transplantation *in vivo*.

#### Example 5

The following Example was published after the priority date of the present application by Roncarolo and Bacchetta (1992) in Bone Marrow  
20 Transplantation: Proceedings of Foetal and Neonatal Cell Transformation and Retroviral Gene Therapy vol. 9, supplement 1. It provides *in vivo* evidence for the importance of T cell repertoire in tolerance after fetal stem cell transplantation.

The T cell repertoire and the mechanism of tolerance was studied in  
25 two patients with severe combined immunodeficiency transplanted with HLA mismatched fetal liver stem cells. They are 18 and 6 years old (as of 1993) and healthy, and show normal immunoresponses to recall antigens. Their T cells are of donor origin, whereas monocytes and B cells remained of the host. The NK cells have different sources since in one patient they  
30 derive from the donor and in the other one from the host. Despite the HLA mismatch between donor and host cells, no acute or chronic graft-versus-host disease was observed. *In vitro* experiments with PBMC showed specific nonresponsiveness for the HLA antigens expressed by the host

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cells. However, an extensive clonal analysis showed that CD4<sup>+</sup> and CD8<sup>+</sup> host-reactive T cell clones recognizing class II and class I HLA molecules of the host, respectively, were present in the peripheral blood of both patients. Limiting dilution experiments indicated that the frequency of CD8<sup>+</sup> host-reactive cells was in the same range as that observed for alloreactive T cells. In contrast, no donor-reactive CD8<sup>+</sup> T cells could be isolated. Host-reactive CD4<sup>+</sup> and CD8<sup>+</sup> T cell clones were normal in their capacity to produce IL-2, IFN- $\gamma$ , GM-CSF, and IL-5, but they failed completely to synthesize IL-4. In addition, CD4<sup>+</sup> T cell clones from patient RV secreted very high levels of IL-10. Interestingly, exogenous IL-10 was able to inhibit the proliferative responses of the CD4<sup>+</sup> host-reactive T cell clones. These data demonstrate that host-reactive cells are not deleted from the donor T cell repertoire following allogeneic fetal liver stem cell transplantation. Therefore, *in vivo* tolerance between the host and the donor is maintained by a peripheral autoregulatory mechanism in which cytokines may play a role.

Bone marrow transplantation from an HLA-identical donor is the therapy of choice for children with severe combined immunodeficiency (SCID). However, transplantation of hemopoietic fetal liver cells (FLT) from a mismatched donor can give sustained engraftment and offer a possible cure in the absence of an HLA-identical marrow donor. See, e.g., Touraine et al. (1987) Thymus 10:75-\_. In such patients, immunocompetent T lymphocytes of donor origin, as determined by HLA typing, could be identified within 2-3 months post-transplantation. Although peripheral T lymphocyte chimerism is routinely detected in SCID children transplanted with fetal liver stem cells, lack of B lymphocyte chimerism is not uncommon. The immunologic evaluation of these patients offers a unique possibility to study human T cell differentiation and self/nonself discrimination under *in vivo* conditions of HLA mismatch between T precursor cells and differentiation environment.

This report on two SCID children transplanted 18 and 6 years ago with fetal liver stem cells from fully HLA-disparate donors provides evidence for *in vivo* activity of IL-10 in regulating the immune system, and

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in particular that IL-10 produced by host-reactive cells may play an important role in down-regulating their responses *in vivo*.

Patient SP received two fetal liver stem cell transplantations with simultaneous injection of syngeneic fetal thymus. Although standard HLA  
 5 typing showed engraftment of cells only from the second donor, a more precise cytofluorometric analysis, using monoclonal antibodies specific for polymorphic HLA determinants, indicated that 10-20% of the T lymphocytes were actually from the first donor. The second patient, RV,  
 10 received seven fetal liver stem cell transplantations, but only one donor-cell population could be identified in the peripheral blood. See Roncarolo et al. (1986) J. Clin. Investig. 77:673-\_\_\_\_\_.

TABLE 3

	HLA TYPING				
	A	C	B	DR	DQ
<u>SP</u>					
Recipient	3-33	6	14-47	4-5	3
1st donor	2-11	4	27-62	1-8	1
2nd donor	1-2	7	8-18	3-9	3
<u>RV</u>					
Recipient	2-13	4-7	x-62	8-10	4-5
Donor	2-30	4	8-35	11-13	6-7

15 In such patients, sustained engraftment of donor T cells was observed after transplantation, whereas B cells and monocytes were of host origin.

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TABLE 4. CHIMERISM

$\alpha\beta$ TCR <sup>+</sup> T cells	
$\gamma\delta$ TCR <sup>+</sup> T cells	Donor origin
Monocytes	
B cells	Host origin
NK cells	Host origin-> Patient SP Donor origin-> Patient RV

Despite this state of split chimerism within cells of the immune system, complete reconstitution was achieved and normal *in vivo* and *in vitro* antibody responses to recall antigens were observed. This is due to the ability of donor T cells to cooperate with the antigen-presenting cells (APC) of the host, across the allogeneic barrier. In particular, tetanus toxoid specific T cell clones of donor origin, isolated from the peripheral blood of patient SP, could recognize the antigen (Ag) processed and presented by host B cells, EBV-transformed B cell lines, and NK cell clones. In contrast, none of the Ag-specific T cell clones tested so far were restricted by the class II HLA antigens expressed by the donor cells. See Roncarolo et al. (1988) J. Expt'l Med. 167:1523-\_\_\_\_; Roncarolo et al. (1991) J. Immunol. 147:781-\_\_\_\_.

The chimerism in the NK population differed in the two patients (Table 4). In one patient fresh NK cells and NK cell clones showed the HLA phenotype of the host; in the other patient they were of donor origin. These NK cells expressed the CD16 CD56 antigens and displayed normal cytotoxic activity against a variety of NK sensitive targets. These findings suggest that the presence of host or donor functional NK cells does not prevent stable engraftment of donor T cells after fetal stem cell transplantation. Despite the coexistence of lymphoid cells with major

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and/or minor histocompatibility antigen differences, complete tolerance was achieved *in vivo* in these two patients, and no signs of acute or chronic graft-versus-host disease were observed. Furthermore, *in vitro* studies showed that specific nonresponsiveness by the donor T cells

5 towards the HLA antigens expressed by the host was present in a primary mixed leucocyte culture, whereas the proliferative responses against allogeneic cells were normal. At the clonal level, however, the findings have differed. Host-reactive cytotoxic T cell clones of donor origin recognizing either HLA class I or HLA class II antigens have been derived

10 from the peripheral blood of both patients. In contrast to what has been reported in SCID patients transplanted with marrow from HLA-haplo-identical parental donors, no donor-reactive T cell clones could be isolated in these two patients. Furthermore, in patient RV no T cell clones specific for the HLA class I locus A antigens that were shared by the host could be

15 identified. Frequency analysis using a modified limiting dilution assay (see Vandekerckhove et al. (1992) J. Expl. Med. 175:1033-1043) confirmed the lack of donor reactivity and demonstrated that the frequency of CD8<sup>+</sup> host-reactive T cells was in the same range as the frequency of T cells reacting against third party HLA antigens. These findings demonstrate that host-

20 reactive cells are not clonally deleted from the donor T cell repertoire. Evidently, such host-reactive T cells are under regulation, since clinical manifestations of graft-versus-host disease were not evident in these patients. It is possible that the host-reactive cells are anergic *in vivo* and that *in vitro* stimulation in the presence of IL-2 can break this anergy.

25 Host-reactive T cells display a peculiar pattern of lymphokine production after polyclonal and antigen-specific stimulation. None of the CD4<sup>+</sup> or CD8<sup>+</sup> T cell clones are able to secrete IL-4, whereas they synthesize normal levels of IL-2, IL-5, and GM-CSF. IFN- $\gamma$  production by these clones is usually very high. In addition, IL-10 production by CD4<sup>+</sup>

30 host-reactive clones of patient RV is extremely high after antigen-specific stimulation and seems to be inversely correlated to the low IL-2 synthesis. Furthermore, addition of exogenous IL-10 can significantly suppress the proliferative responses of CD4<sup>+</sup> host-reactive T cell clones *in vitro*. This provides evidence that IL-10 production by host-reactive cells may play an

35 important role in down-regulating their responses *in vivo*.

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Example 6

This example investigates IL-10 production in SCID patients transplanted with allogeneic stem cells. Children with Severe Combined Immunodeficiency (SCID) can be transplanted with HLA mismatched fetal stem cells and immunological reconstitution is obtained also when only the T cells of the donor engraft. Despite tolerance to the host, host-reactive T cells are still present at high frequencies in the peripheral blood of these patients suggesting that an autoregulatory suppressor mechanism may be responsible for the in vivo homeostasis. IL-10 has recently been described as a suppressive cytokine able to prevent activation and proliferation of (allo)antigen specific T cells. Semiquantitative PCR on total peripheral blood mononuclear cells from two patient indicated that the IL-10 mRNA levels were much higher than those of normal donors, whereas IFN- $\gamma$  and GM-CSF mRNAs were comparable. PCR analysis on purified monocytes, B and T cells demonstrated that the monocytes of host origin were responsible for the enhanced IL-10 production. In one patient, high levels of IL-10 mRNA were also observed in purified total T cells. Furthermore, high levels of IL-10 production by CD4<sup>+</sup> host reactive T cells suppressed in an autoregulatory fashion the proliferation of these cells in response to host cells. These results suggest that high endogenous IL-10 production may account for the suppression of allo reactivity after stem cell transplantation.

On December 20th 1989, Applicants deposited separate cultures of *E. coli* MC1061 carrying pH5C, pH15C, and pBCRF1(SR $\alpha$ ) with the American Type Culture Collection, Rockville, MD, USA (ATCC), under accession numbers 68191, 68192, and 68193, respectively. These deposits were made under conditions as provided under ATCC's agreement for Culture Deposit for Patent Purposes, which assures that the deposit will be made available to the US Commissioner of Patents and Trademarks pursuant to 35 USC 122 and 37 CFR 1.14, and will be made available to the public upon issue of a U.S. patent, which requires that the deposit be maintained. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the



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rights granted under the authority of any government in accordance with its patent laws.

The Deposits have been modified to satisfy the requirements of the Budapest Convention.

- 5           The descriptions of the foregoing embodiments of the invention have been presented for purpose of illustration and description. They are not intended to be exhaustive or to limit the invention to the precise forms disclosed, and obviously many modifications and variations are possible in ~~light of the above teaching. The embodiments were chosen and described~~
- 10 in order to best explain the principles of the invention to thereby enable others skilled in the art to best utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated. It is intended that the scope of the invention be defined by the claims appended hereto.

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SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 178 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

10 (A) ORGANISM: Homo sapiens

(ix) FEATURE:

(D) OTHER INFORMATION: Human IL-10 (human cytokine  
 synthesis inhibitory factor, human CSIF)

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

	Met	His	Ser	Ser	Ala	Leu	Leu	Cys	Cys	Leu	Val	Leu	Leu	Thr	Gly	15
					5					10						
	Val	Arg	Ala	Ser	Pro	Gly	Gln	Gly	Thr	Gln	Ser	Glu	Asn	Ser	Cys	30
					20					25						
20	Thr	His	Phe	Pro	Gly	Asn	Leu	Pro	Asn	Met	Leu	Arg	Asp	Leu	Arg	45
					35					40						
	Asp	Ala	Phe	Ser	Arg	Val	Lys	Thr	Phe	Phe	Gln	Met	Lys	Asp	Gln	60
					50					55						
	Leu	Asp	Asn	Leu	Leu	Leu	Lys	Glu	Ser	Leu	Leu	Glu	Asp	Phe	Lys	75
25					65					70						
	Gly	Tyr	Leu	Gly	Cys	Gln	Ala	Leu	Ser	Glu	Met	Ile	Gln	Phe	Tyr	90
					80					85						
	Leu	Glu	Glu	Val	Met	Pro	Gln	Ala	Glu	Asn	Gln	Asp	Pro	Asp	Ile	105
					95					100						
30	Lys	Ala	His	Val	Asn	Ser	Leu	Gly	Glu	Asn	Leu	Lys	Thr	Leu	Arg	120
					110					115						
	Leu	Arg	Leu	Arg	Arg	Cys	His	Arg	Phe	Leu	Pro	Cys	Glu	Asn	Lys	135
					125					130						
	Ser	Lys	Ala	Val	Glu	Gln	Val	Lys	Asn	Ala	Phe	Asn	Lys	Leu	Gln	150
35					140					145						
	Glu	Lys	Gly	Ile	Tyr	Lys	Ala	Met	Ser	Glu	Phe	Asp	Ile	Phe	Ile	165
					155					160						
	Asn	Tyr	Ile	Glu	Ala	Tyr	Met	Thr	Met	Lys	Ile	Arg	Asn			
					170					175						

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## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 170 amino acids

5 (B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

(A) ORGANISM: B95-8 Epstein-Barr Virus

10 (ix) FEATURE:

(D) OTHER INFORMATION: Viral IL-10 (BCRF1)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

	Met	Glu	Arg	Arg	Leu	Val	Val	Thr	Leu	Gln	Cys	Leu	Val	Leu	Leu	
					5					10					15	
15	Tyr	Leu	Ala	Pro	Glu	Cys	Gly	Gly	Thr	Asp	Gln	Cys	Asp	Asn	Phe	
					20					25					30	
	Pro	Gln	Met	Leu	Arg	Asp	Leu	Arg	Asp	Ala	Phe	Ser	Arg	Val	Lys	
					35					40					45	
20	Thr	Phe	Phe	Gln	Thr	Lys	Asp	Glu	Val	Asp	Asn	Leu	Leu	Leu	Lys	
					50					55					60	
	Glu	Ser	Leu	Leu	Glu	Asp	Phe	Lys	Gly	Tyr	Leu	Gly	Cys	Gln	Ala	
					65					70					75	
	Leu	Ser	Glu	Met	Ile	Gln	Phe	Tyr	Leu	Glu	Glu	Val	Met	Pro	Gln	
					80					85					90	
25	Ala	Glu	Asn	Gln	Asp	Pro	Glu	Ala	Lys	Asp	His	Val	Asn	Ser	Leu	
					95					100					105	
	Gly	Glu	Asn	Leu	Lys	Thr	Leu	Arg	Leu	Arg	Leu	Arg	Arg	Cys	His	
					110					115					120	
30	Arg	Phe	Leu	Pro	Cys	Glu	Asn	Lys	Ser	Lys	Ala	Val	Glu	Gln	Ile	
					125					130					135	
	Lys	Asn	Ala	Phe	Asn	Lys	Leu	Gln	Glu	Lys	Gly	Ile	Tyr	Lys	Ala	
					140					145					150	
	Met	Ser	Glu	Phe	Asp	Ile	Phe	Ile	Asn	Tyr	Ile	Glu	Ala	Tyr	Met	
					155					160					165	
35	Thr	Ile	Lys	Ala	Arg											
					170											

## (2) INFORMATION FOR SEQ ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 160 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: protein  
 5 (ix) FEATURE:  
 (D) OTHER INFORMATION: Mature human IL-10 (human cytokine synthesis inhibitory factor, human CSIF)  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

10	Ser	Pro	Gly	Gln	Gly	Thr	Gln	Ser	Glu	Asn	Ser	Cys	Thr	His	Phe	5	10	15
	Pro	Gly	Asn	Leu	Pro	Asn	Met	Leu	Arg	Asp	Leu	Arg	Asp	Ala	Phe	20	25	30
15	Ser	Arg	Val	Lys	Thr	Phe	Phe	Gln	Met	Lys	Asp	Gln	Leu	Asp	Asn	35	40	45
	Leu	Leu	Leu	Lys	Glu	Ser	Leu	Leu	Glu	Asp	Phe	Lys	Gly	Tyr	Leu	50	55	60
	Gly	Cys	Gln	Ala	Leu	Ser	Glu	Met	Ile	Gln	Phe	Tyr	Leu	Glu	Glu	65	70	75
20	Val	Met	Pro	Gln	Ala	Glu	Asn	Gln	Asp	Pro	Asp	Ile	Lys	Ala	His	80	85	90
	Val	Asn	Ser	Leu	Gly	Glu	Asn	Leu	Lys	Thr	Leu	Arg	Leu	Arg	Leu	95	100	105
25	Arg	Arg	Cys	His	Arg	Phe	Leu	Pro	Cys	Glu	Asn	Lys	Ser	Lys	Ala	110	115	120
	Val	Glu	Gln	Val	Lys	Asn	Ala	Phe	Asn	Lys	Leu	Gln	Glu	Lys	Gly	125	130	135
	Ile	Tyr	Lys	Ala	Met	Ser	Glu	Phe	Asp	Ile	Phe	Ile	Asn	Tyr	Ile	140	145	150
30	Glu	Ala	Tyr	Met	Thr	Met	Lys	Ile	Arg	Asn						155	160	

- (2) INFORMATION FOR SEQ ID NO: 4:  
 (i) SEQUENCE CHARACTERISTICS:  
 35 (A) LENGTH: 147 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: protein  
 (ix) FEATURE:

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(D) OTHER INFORMATION: Mature viral IL-10 (BCRF1)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

	Thr	Asp	Gln	Cys	Asp	Asn	Phe	Pro	Gln	Met	Leu	Arg	Asp	Leu	Arg	
					5					10					15	
5	Asp	Ala	Phe	Ser	Arg	Val	Lys	Thr	Phe	Phe	Gln	Thr	Lys	Asp	Glu	
					20					25					30	
	Val	Asp	Asn	Leu	Leu	Leu	Lys	Glu	Ser	Leu	Leu	Glu	Asp	Phe	Lys	
					35					40					45	
10	Gly	Tyr	Leu	Gly	Cys	Gln	Ala	Leu	Ser	Glu	Met	Ile	Gln	Phe	Tyr	
					50					55					60	
	Leu	Glu	Glu	Val	Met	Pro	Gln	Ala	Glu	Asn	Gln	Asp	Pro	Glu	Ala	
					65					70					75	
	Lys	Asp	His	Val	Asn	Ser	Leu	Gly	Glu	Asn	Leu	Lys	Thr	Leu	Arg	
					80					85					90	
15	Leu	Arg	Leu	Arg	Arg	Cys	His	Arg	Phe	Leu	Pro	Cys	Glu	Asn	Lys	
					95					100					105	
	Ser	Lys	Ala	Val	Glu	Gln	Ile	Lys	Asn	Ala	Phe	Asn	Lys	Leu	Gln	
					110					115					120	
20	Glu	Lys	Gly	Ile	Tyr	Lys	Ala	Met	Ser	Glu	Phe	Asp	Ile	Phe	Ile	
					125					130					135	
	Asn	Tyr	Ile	Glu	Ala	Tyr	Met	Thr	Ile	Lys	Ala	Arg				
					140					145						

(2) INFORMATION FOR SEQ ID NO: 5:

25 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30 (ix) FEATURE:

(D) OTHER INFORMATION: As double-stranded  
fragment, encodes a consensus ribosome binding  
site.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

35 GTAAGGAGGT TTAAC

15

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

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(A) LENGTH: 60 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## 5 (ix) FEATURE:

(D) OTHER INFORMATION: T and GGTAC at positions 51 and 56-60 differ from those of the native sequence; together with SEQ ID NO: 7, SEQ ID NO: 6 forms double-stranded Fragment 1A/B of synthetic CSIF gene with 4-base sticky end at positions 57-60.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AGCCCAGGCC AGGGCACCCA GTCTGAGAAC AGCTGCACCC ACTTCCCAGG 50  
TAACCGGTAC 60

15

## (2) INFORMATION FOR SEQ ID NO: 7:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 56 base pairs

(B) TYPE: nucleic acid

20 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ix) FEATURE:

(D) OTHER INFORMATION: C and A at positions 1 and 6 differ from those of the native sequence; together with SEQ ID NO: 6, SEQ ID NO: 7 forms double-stranded Fragment 1A/B of synthetic CSIF gene.

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CGGTTACCTG GGAAGTGGGT GCAGCTGTTC TCAGACTGGG TGCCCTGGCC 50  
30 TGGGCT 56

## (2) INFORMATION FOR SEQ ID NO: 8:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 62 base pairs

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(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:

- 5 (D) OTHER INFORMATION: T position 2 differs from  
that of the native sequence; together with SEQ  
ID NO: 9, SEQ ID NO: 8 forms double-stranded  
Fragment 2A/B of synthetic CSIF gene with 5- and  
9-base sticky ends at positions 1-5 and 54-62.

- 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GTAACCTGCC TAACATGCTT CGAGATCTCC GAGATGCCTT CAGCAGAGTG 50  
AAGACTTTCT TT 62

(2) INFORMATION FOR SEQ ID NO: 9:

- 15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 48 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

- 20 (ix) FEATURE:

(D) OTHER INFORMATION: Together with SEQ ID NO: 8,  
SEQ ID NO: 9 forms double-stranded Fragment 2A/B of  
synthetic CSIF gene.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

25 CTTCACTCTG CTGAAGGCAT CTCGGAGATC TCGAAGCATG TTAGGCAG 48

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs

- 30 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:

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(D) OTHER INFORMATION: C and T at positions 30 and 32 differ from those of the native sequence; together with SEQ ID NO: 11, SEQ ID NO: 10 forms double-stranded Fragment 3A/B of synthetic CSIF gene.

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CAAATGAAGG ATCAGCTGGA CAACTTGTTT TTAAG

35

(2) INFORMATION FOR SEQ ID NO: 11:

10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44 base pairs

15

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20

(ix) FEATURE:

(D) OTHER INFORMATION: A and G at positions 4 and 6 differ from those of the native sequence; together with SEQ ID NO: 10, SEQ ID NO: 11 forms double-stranded Fragment 3A/B of synthetic CSIF gene with 9-base sticky end at positions 36-44.

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CTTAAGAACA AGTTGTCCAG CTGATCCTTC ATTTGAAAGA AAGT

44

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

35



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(A) LENGTH: 69 base pairs

(B) TYPE: nucleic acid

5 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:

10

(D) OTHER INFORMATION: T at position 69 differs from that of the native sequence; together with SEQ ID NO: 13, SEQ ID NO: 12 forms double-stranded Fragment 4A/B of synthetic CSIF gene.

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GAGTCCTTGC TGGAGGACTT TAAGGGTTAC CTGGGTTGCC AAGCCTTGTC 50  
TGAGATGATC CAGTTTTAT 69

20 (2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 73 base pairs

25

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

30

(D) TOPOLOGY: linear

(ix) FEATURE:

(D) OTHER INFORMATION: T and A at positions 2 and 35 5 differ from those of the native sequence; together with

- 40 -

SEQ ID NO: 12, SEQ ID NO: 13 forms double-stranded  
Fragment 4A/B of synthetic CSIF gene with 4-base sticky  
end at positions 1-4.

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CTAGATAAAA CTGGATCATC TCAGACAAGG CTTGGCAACC CAGGTAACCC 50  
TTAAAGTCCT CCAGCAAGGA CTC 73

(2) INFORMATION FOR SEQ ID NO: 14:

10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 61 base pairs

15 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20

(ix) FEATURE:

(D) OTHER INFORMATION: A, T and G at positions 3,  
57 and 61 differ from those of the native sequence;  
25 together with SEQ ID NO: 15, SEQ ID NO: 14 forms double-  
stranded Fragment 5A/B of synthetic CSIF gene.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CTAGAGGAGG TGATGCCCCA AGCTGAGAAC CAAGACCCAG ACATCAAGGC 50  
30 GCATGTTAAC G 61

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

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(A) LENGTH: 65 base pairs

(B) TYPE: nucleic acid

5

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

10 (ix) FEATURE:

(D) OTHER INFORMATION: TCGAC, A and T at positions  
1-5, 9 and 63 differ from those of the native sequence;  
together with SEQ ID NO: 14, SEQ ID NO: 15 forms double-  
15 stranded Fragment 5A/B of synthetic CSIF gene with 4-base  
sticky end at positions 1-4.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

TCGACGTTAA CATGCGCCTT GATGTCTGGG TCTTGGTTCT CAGCTTGGGG 50  
20 CATCACCTCC TCTAG 65

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

25

(A) LENGTH: 63 base pairs

(B) TYPE: nucleic acid

30

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:

35

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(D) OTHER INFORMATION: CTGCA at positions 58-63 differ from those of the native sequence; together with SEQ ID NO: 17, SEQ ID NO: 16 forms double-stranded Fragment 6A/B of synthetic CSIF gene with 4-base sticky end at positions 59-63.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

AACTCCCTGG GGGAGAACCT GAAGACCCTC AGGCTGAGGC TACGGCGCTG 50  
TCATCGATCT GCA 63

10

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 59 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:

(D) OTHER INFORMATION: G at position 1 differs from that of the native sequence; together with SEQ ID NO: 16, SEQ ID NO: 17 forms double-stranded Fragment 6A/B of synthetic CSIF gene.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GATCGATGAC AGCGCCGTAG CCTCAGCCTG AGGGTCTTCA GGTCTCTCCC 50  
CAGGGAGTT 59

(2) INFORMATION FOR SEQ ID NO: 18:

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## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- 10 (D) TOPOLOGY: linear

## (ix) FEATURE:

- (D) OTHER INFORMATION: C, G and GCATG at positions  
15 51, 54 and 56-60 differ from those of the native sequence;  
together with SEQ ID NO: 19, SEQ ID NO: 18 forms double-  
stranded Fragment 7A/B of synthetic CSIF gene with 2- and  
4-base sticky ends at positions 1-2 and 57-60.

## 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CGATTTCTTC CCTGTCAAAA CAAGAGCAAG GCCGTGGAGC AGGTGAAGAA 50  
CGCGTG CATG 60

## (2) INFORMATION FOR SEQ ID NO: 19:

25

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 54 base pairs
- 30 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35

- 44 -

## (ix) FEATURE:

(D) OTHER INFORMATION: C, C and G at positions 1, 3 and 6 differ from those of the native sequence;

5 together with SEQ ID NO: 18, SEQ ID NO: 19 forms double-stranded Fragment 7A/B of synthetic CSIF gene.

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CACGCGTTCT TCACCTGCTC CACGGCCTTG CTCTTGTTTT GACAGGGAAG 50  
10 AAAT 54

## (2) INFORMATION FOR SEQ ID NO: 20:

## (i) SEQUENCE CHARACTERISTICS:

15

(A) LENGTH: 58 base pairs

(B) TYPE: nucleic acid

20

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ix) FEATURE:

25

(D) OTHER INFORMATION: Together with SEQ ID NO: 21, SEQ ID NO: 20 forms double-stranded Fragment 8A/B of synthetic CSIF gene with 4- and 9-base sticky ends at positions 1-4 and 50-58.

30

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CGCGTTTAAT AATAAGCTCC AAGACAAAGG CATCTACAAA GCCATGAGTG 50  
AGTTTGAC 58

- 45 -

## (2) INFORMATION FOR SEQ ID NO: 21:

## (i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 45 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ix) FEATURE:

15 (D) OTHER INFORMATION: Together with SEQ ID NO:  
20, SEQ ID NO: 21 forms double-stranded Fragment 8A/B of  
synthetic CSIF gene.

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

20 ACTCATGGCT TTGTAGATGC CTTTGTCTTG GAGCTTATTA TTAAA 45

## (2) INFORMATION FOR SEQ ID NO: 22:

## (i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 51 base pairs

(B) TYPE: nucleic acid

30 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ix) FEATURE:

35

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(D) OTHER INFORMATION: Together with SEQ ID NO: 23, SEQ ID NO: 22 forms double-stranded Fragment 9A/B of synthetic CSIF gene.

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

ATCTTCATCA ACTACATAGA AGCCTACATG ACAATGAAGA TACGAAACTG A 51

(2) INFORMATION FOR SEQ ID NO: 23:

10 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 64 base pairs

(B) TYPE: nucleic acid

15

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

20 (ix) FEATURE:

(D) OTHER INFORMATION: AGCT at positions 1-4 differ from those of the native sequence; together with SEQ ID NO: 22, SEQ ID NO: 23 forms double-stranded  
25 Fragment 9A/B of synthetic CSIF gene with 4- and 9-base sticky ends at positions 1-4 and 56-64.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

AGCTTCAGTT TCGTATCTTC ATTGTCATGT AGGCTTCTAT GTAGTTGATG 50  
30 AAGATGTCAA ACTC 64

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:



- 47 -

(A) LENGTH: 519 base pairs

(B) TYPE: nucleic acid

5

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

10 (ix) FEATURE:

(D) OTHER INFORMATION: Encodes viral IL-10.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

15	AATTC ATG GAG CGA AGG TTA GTG GTC ACT CTG CAG TGC CTG GTG	44
	CTG CTT TAC CTG GCA CCT GAG TGT GGA GGT ACA GAC CAA TGT	86
	GAC AAT TTT CCC CAA ATG TTG AGG GAC CTA AGA GAT GCC TTC	128
	AGT CGT GTT AAA ACC TTT TTC CAG ACA AAG GAC GAG GTA GAT	170
	AAC CTT TTG CTC AAG GAG TCT CTG CTA GAG GAC TTT AAG GGC	212
20	TAC CTT GGA TGC CAG GCC CTG TCA GAA ATG ATC CAA TTC TAC	254
	CTG GAG GAA GTC ATG CCA CAG GCT GAA AAC CAG GAC CCG GAG	296
	GCT AAG GAC CAT GTC AAT TCT TTG GGT GAA AAT CTA AAG ACC	338
	CTA CGG CTC CGC CTG CGC AGG TGC CAC AGG TTC CTG CCG TGT	380
	GAG AAC AAG AGT AAA GCT GTG GAA CAG ATA AAA AAT GCC TTT	422
25	AAC AAG CTG CAG GAA AAA GGA ATT TAC AAA GCC ATG AGT GAA	464
	TTT GAC ATT TTT ATT AAC TAC ATA GAA GCA TAC ATG ACA ATT	506
	AAA GCC AGG TGA G	519

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CLAIMS:

1. A method of suppressing graft-vs.-host disease or tissue rejection in an individual, the method comprising the step of administering an effective amount of interleukin-10 or an agonist thereof to the individual.
- 5 2. The method of claim 1 wherein said interleukin-10 is selected from the group consisting of viral interleukin-10 and human interleukin-10.
3. A method of suppressing graft-vs.-host disease or tissue rejection in an individual, the method comprising the step of administering an effective amount of interleukin-10 to the individual.
- 10 4. The method of claim 3 wherein said interleukin-10 is selected from the group consisting of viral interleukin-10 and human interleukin-10.
5. A method of suppressing tissue rejection in an individual, the method comprising the step of administering an effective amount of interleukin-10 to the individual.
- 15 6. The method of claim 5 wherein said interleukin-10 is selected from the group consisting of viral interleukin-10 and human interleukin-10.
7. A method as claimed in claim 1 wherein said interleukin-10 is a post-translational variant or mutein.
8. A method of suppressing graft-vs.-host disease or tissue rejection in  
20 an individual, the method comprising the step of administering to the individual an effective amount of interleukin-10 as defined herein in SEQ. ID. NO. 3.
9. The method of claim 8 wherein said interleukin-10 is selected from the group consisting of viral interleukin-10 and human interleukin-10.
- 25 10. The use of interleukin-10 for treating graft-vs.-host disease or tissue rejection.
11. The use of interleukin-10 for the manufacture of a medicament for the treatment of graft-vs.-host disease or tissue rejection.

– 49 –

12. A method of treating a patient having graft-vs.-host disease comprising the administration to said patient of an effective amount of interleukin-10.
13. Interleukin-10 for treating graft-vs.-host disease or tissue rejection.
- 5 14. The use of a pharmaceutical composition containing interleukin-10 for treating graft-vs.-host disease or tissue rejection.
15. ~~The use of interleukin-10 for preparing a pharmaceutical~~ composition useful for treating graft-vs.-host disease or tissue rejection.

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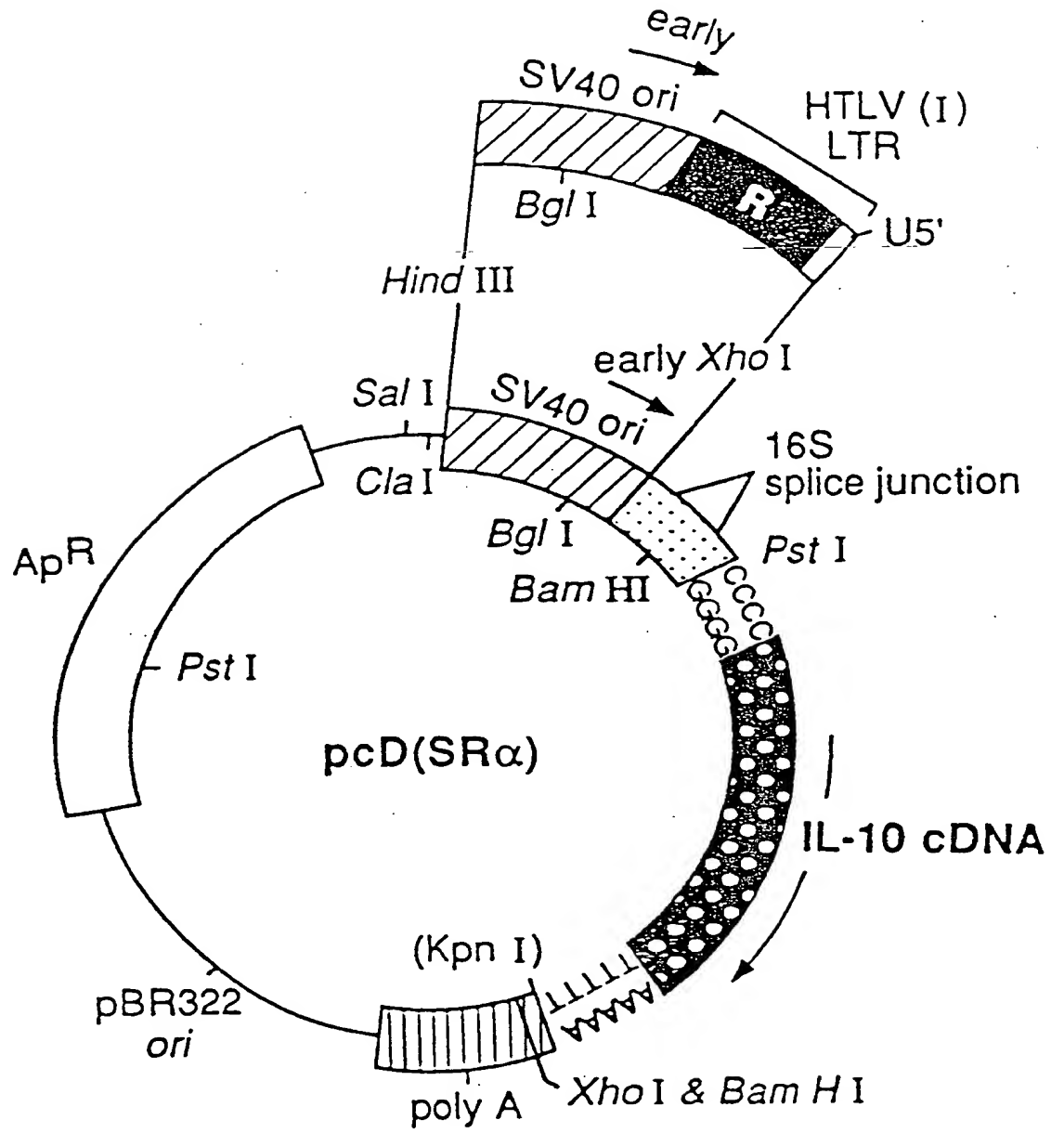
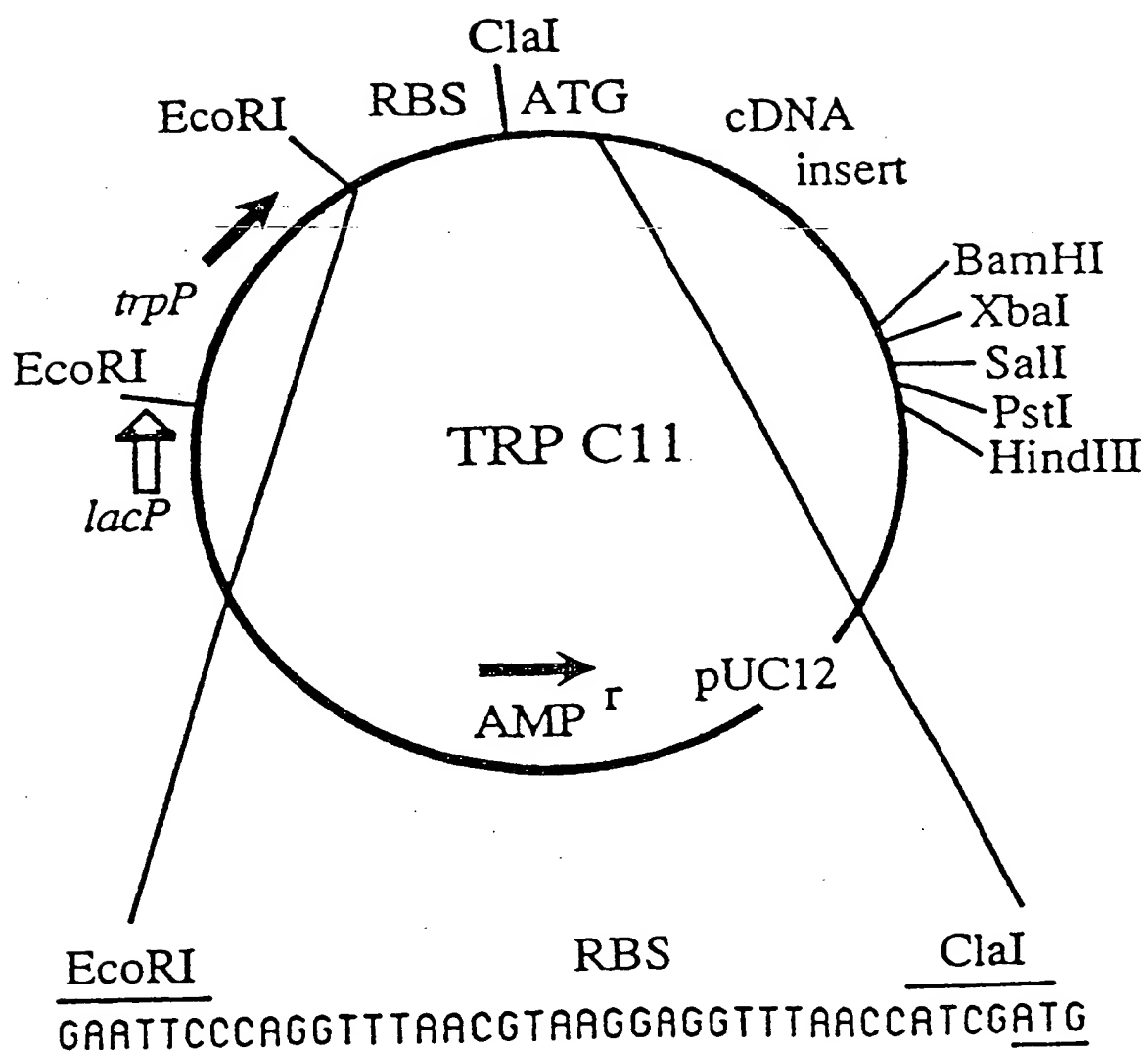


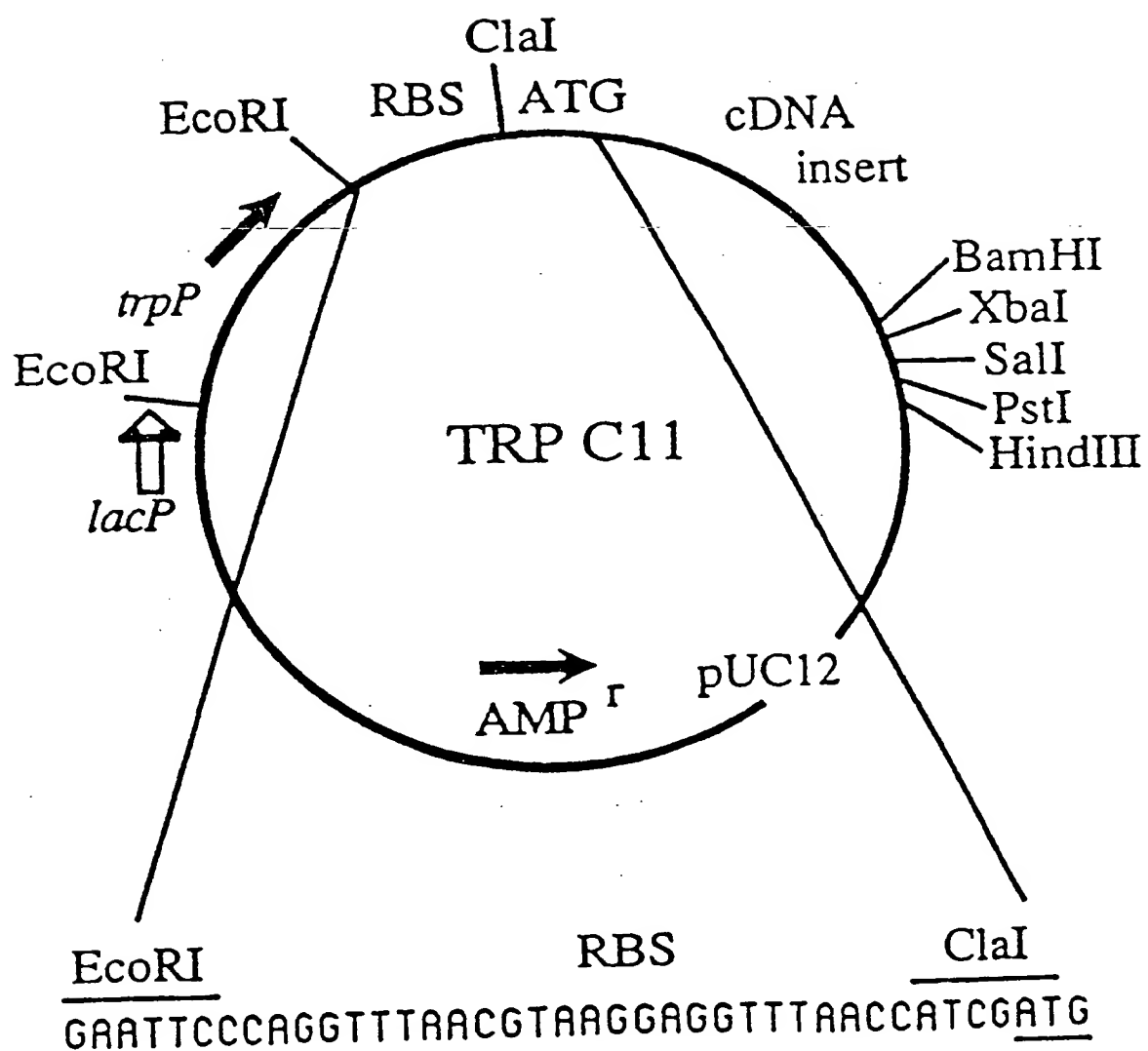
Figure 1

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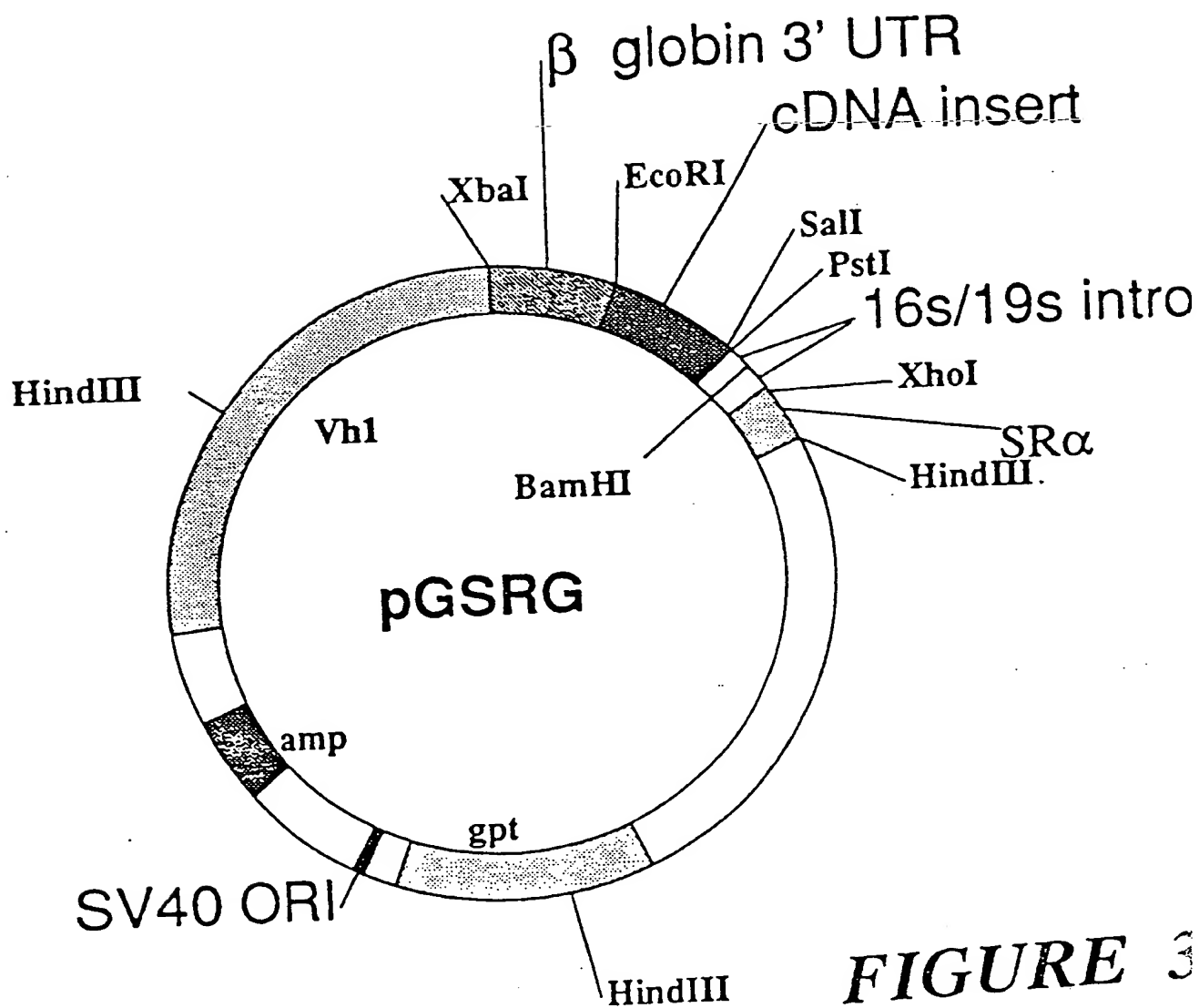
**FIGURE 2**

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**FIGURE 2**

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**FIGURE 3**

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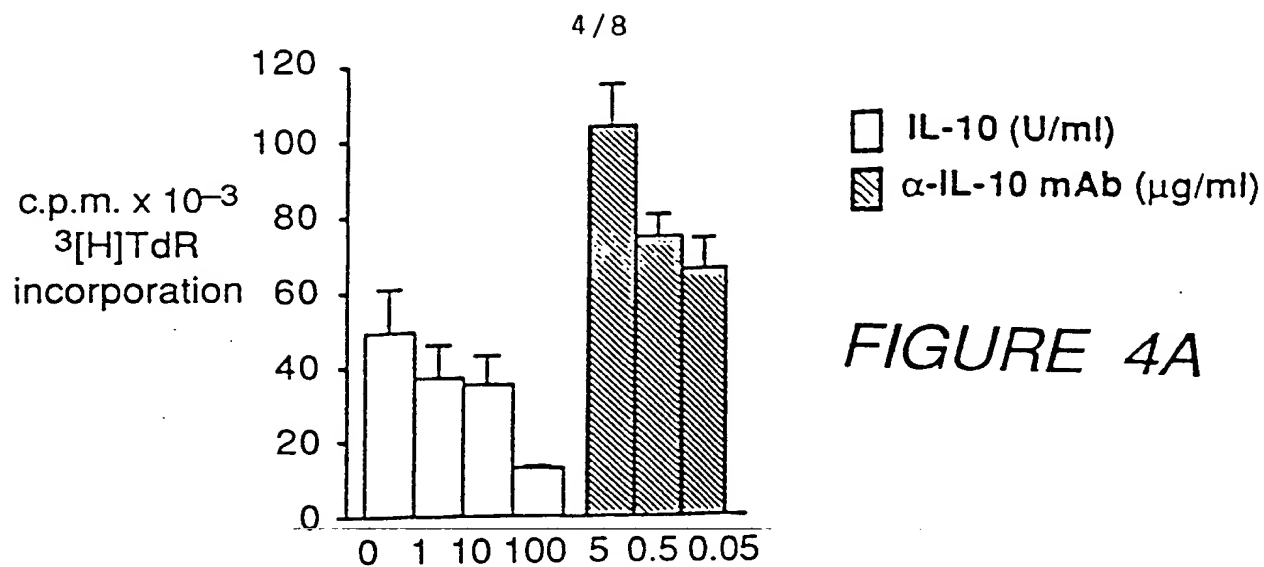


FIGURE 4A

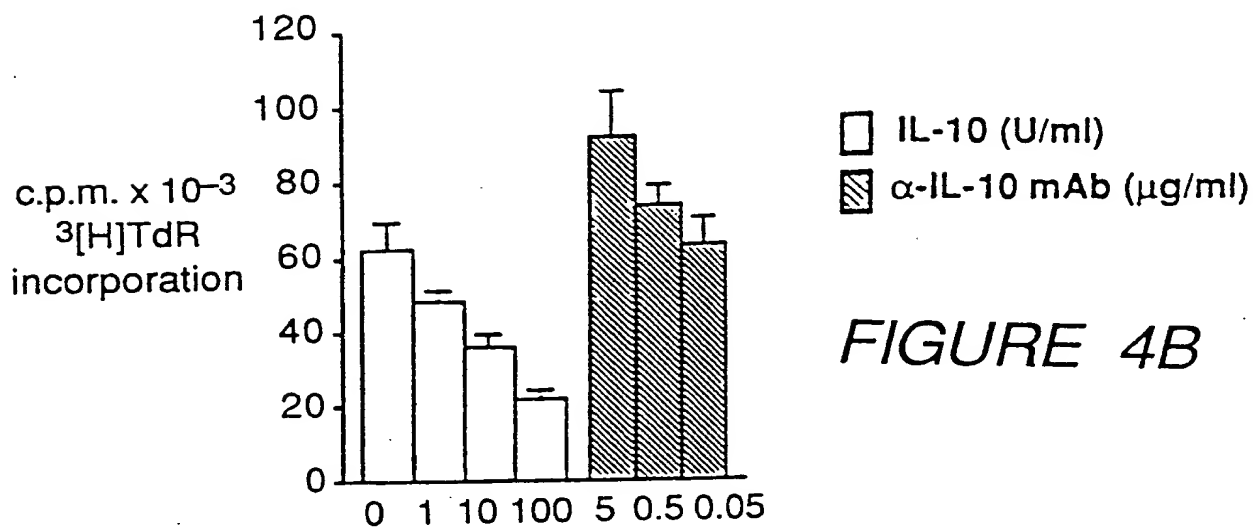


FIGURE 4B

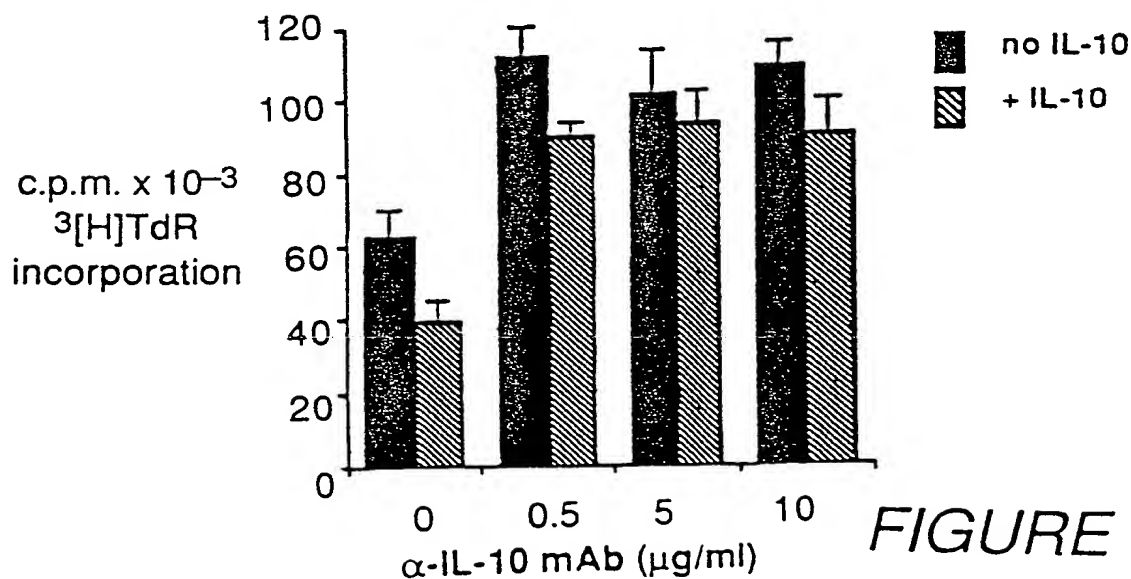
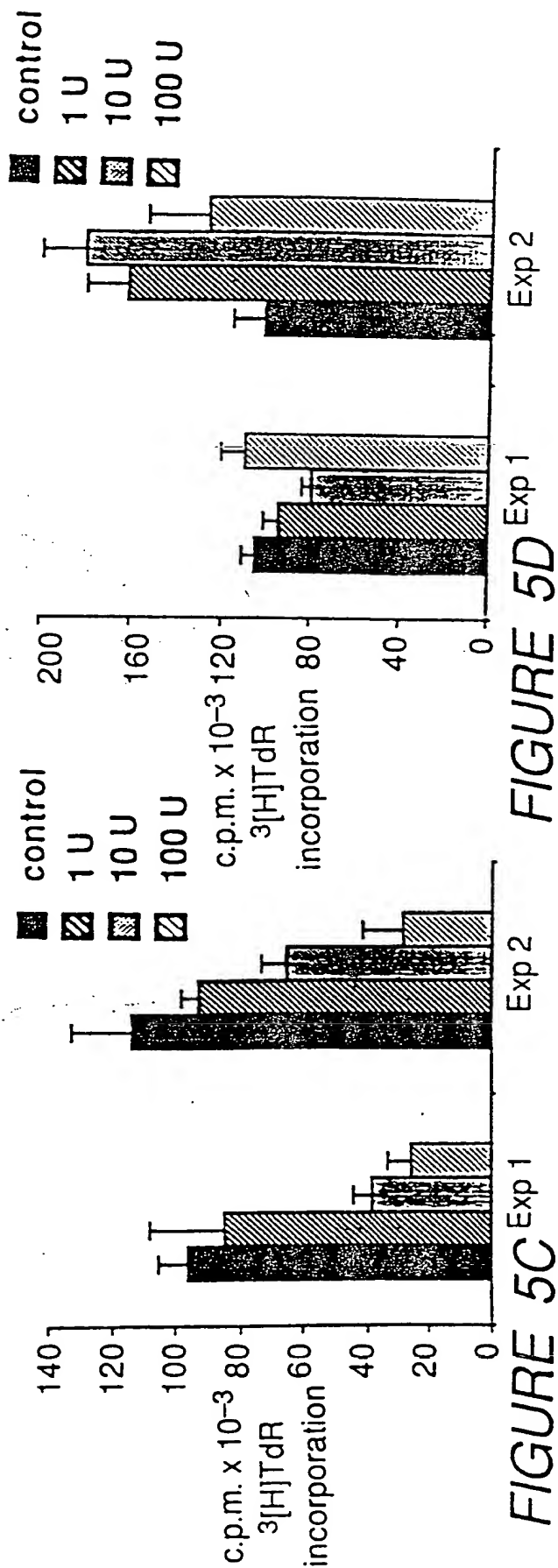
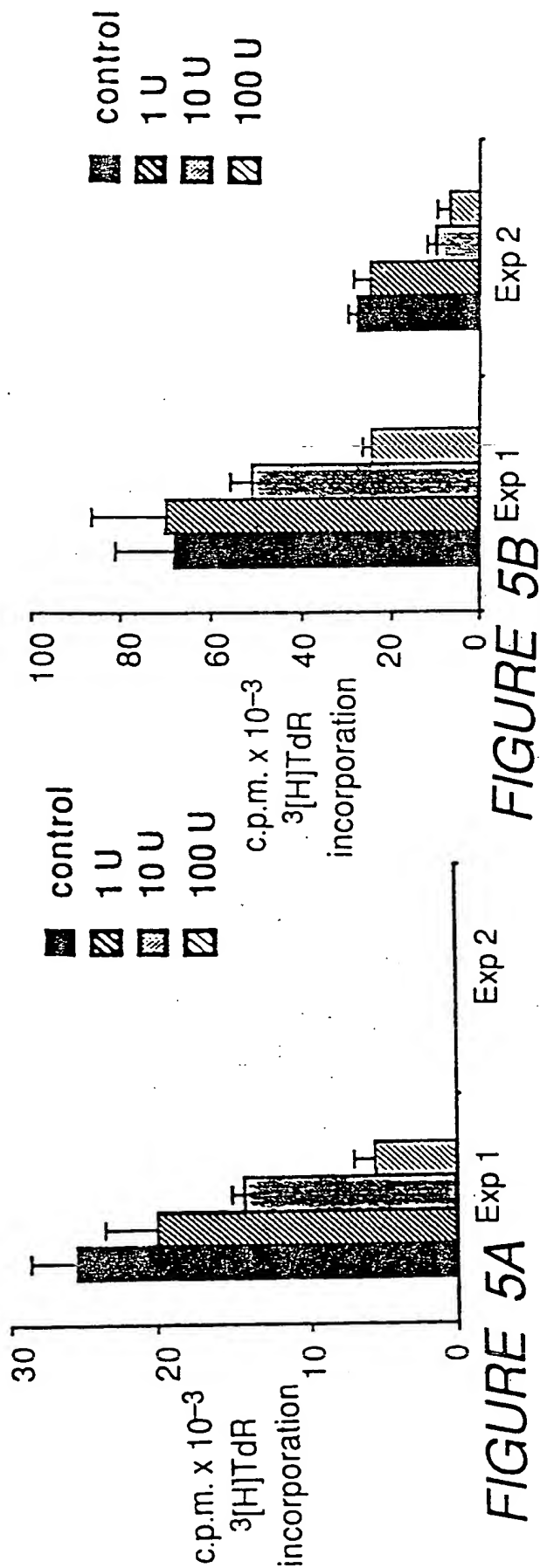


FIGURE 4C

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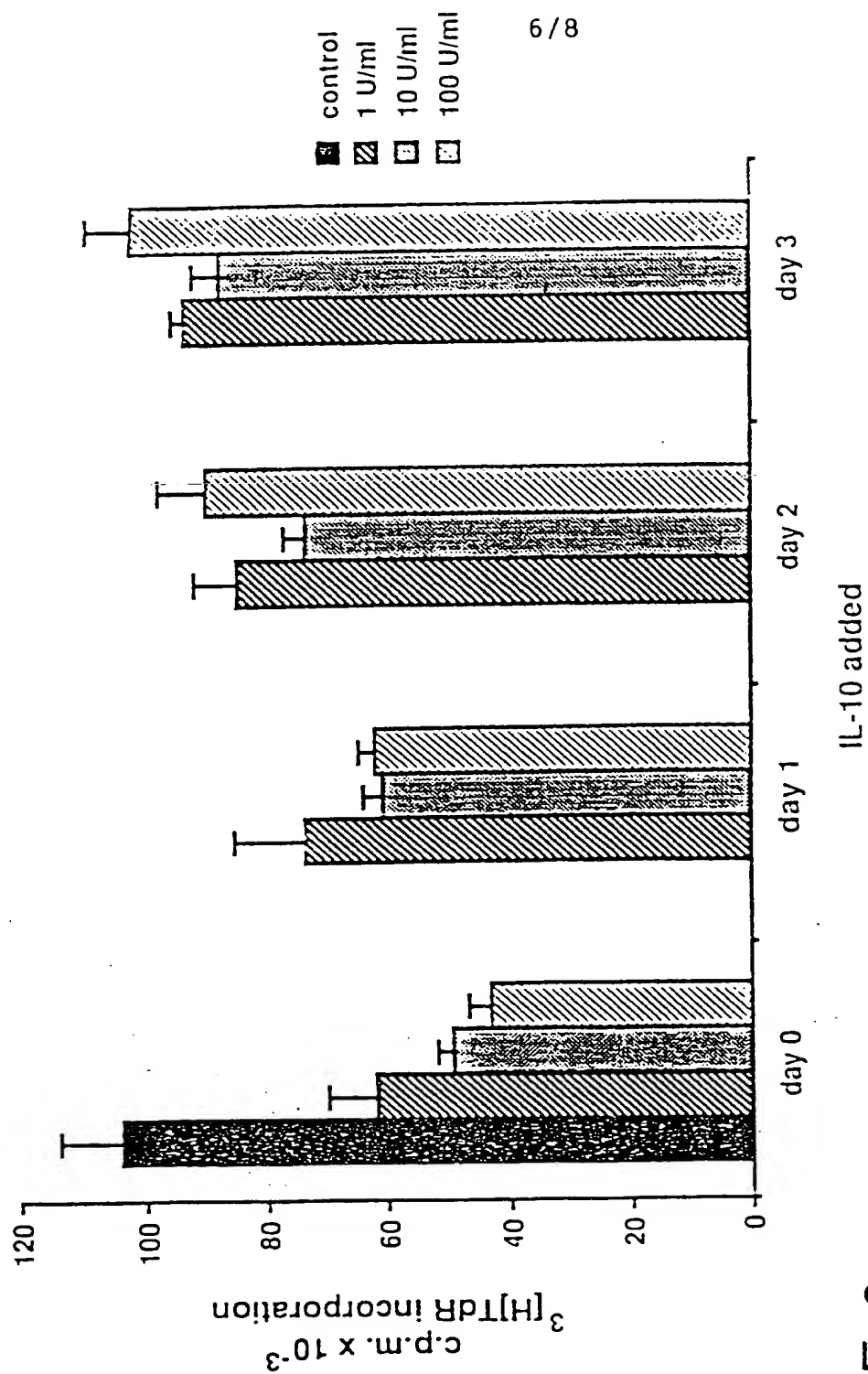


FIGURE 6

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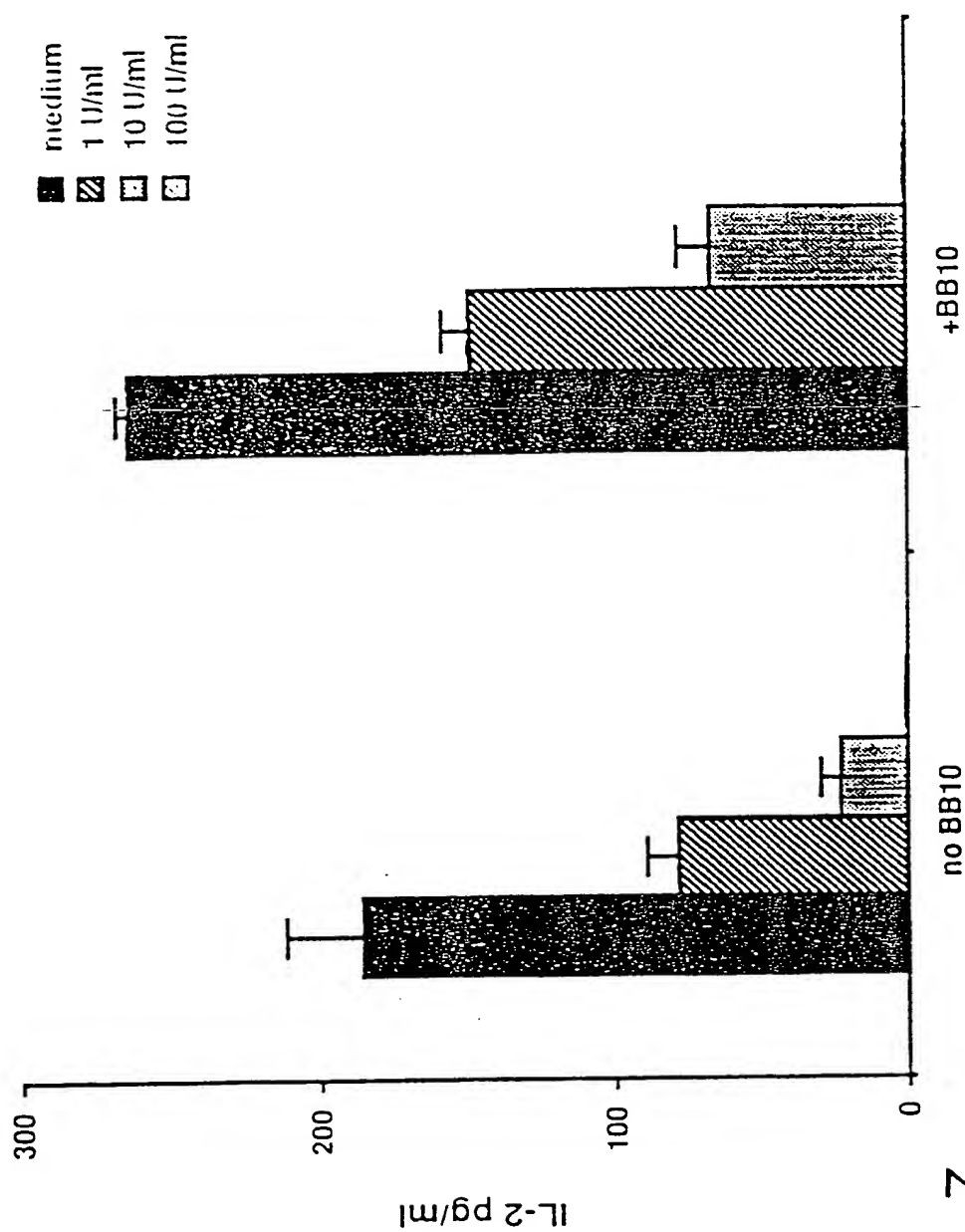
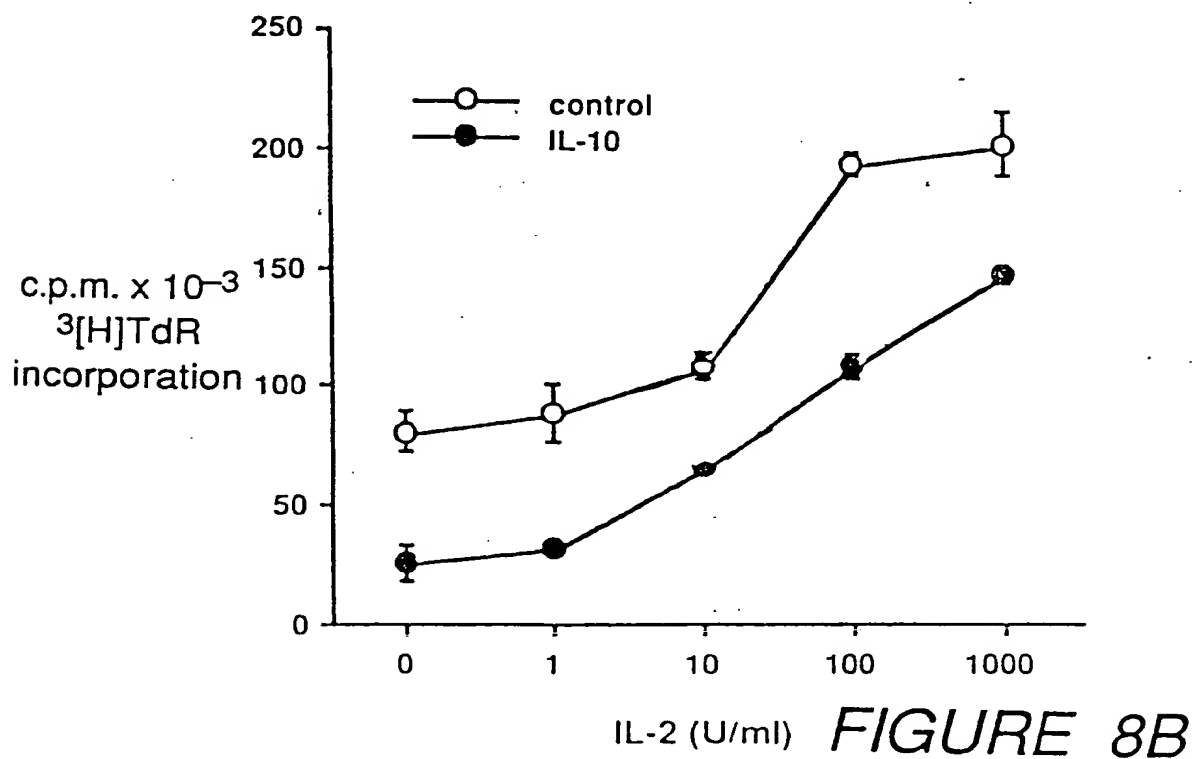
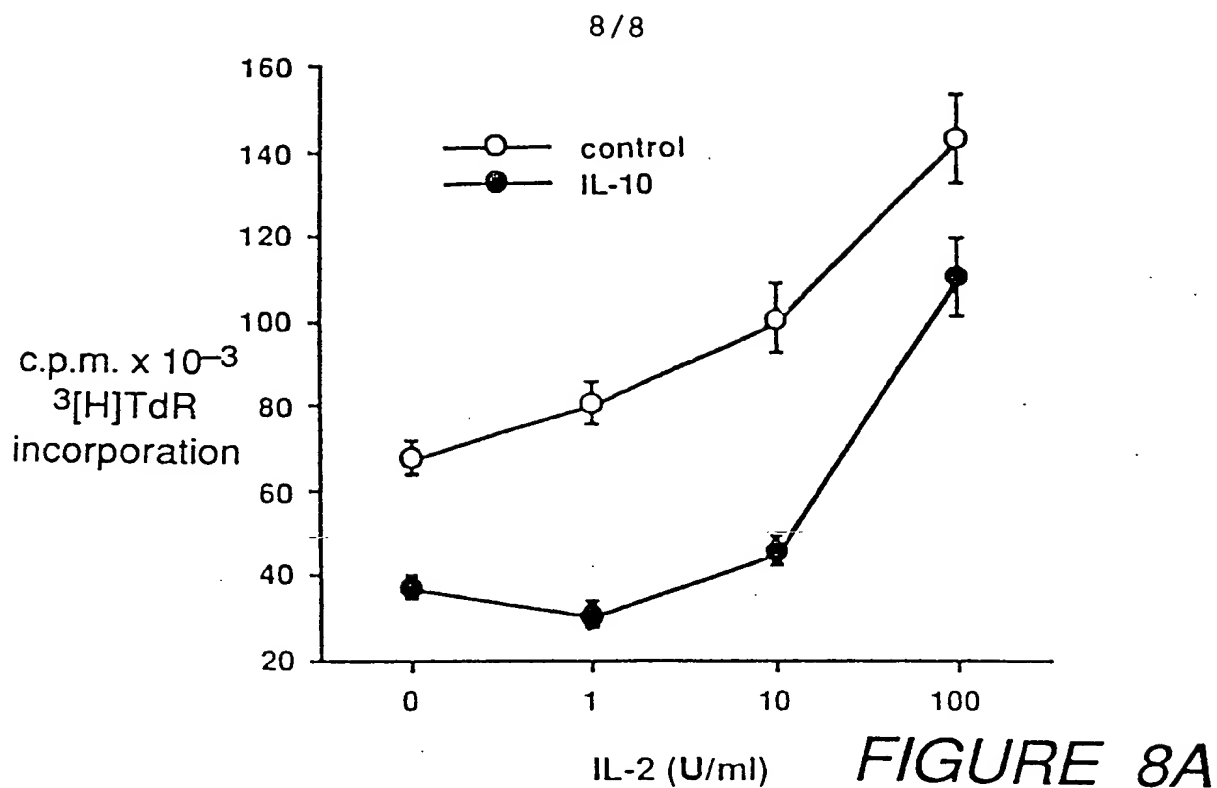


FIGURE 7

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/01665

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl. 5 A61K37/02; //C12N15/24, C07K13/00		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	A61K ; C07K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	EP,A,0 405 980 (SCHERING CORPORATION) 2 January 1991 see page 2, line 48; claims 1-4,17 ---	1-15
X,0	JOURNAL OF CELLULAR BIOCHEMISTRY (KEystone SYMPOSIUM ON BONE MARROW TRANSPLANTATION, KEystone, COLORADO, US, 19-26 JANUARY, 1992) vol. SUPPL, no. 16A, 1992, NEW YORK, N.Y., US page 214 M-G. RONCAROLO ET AL. 'SCID PATIENTS RECONSTITUTED BY FETAL LIVER STEM CELLS: POSSIBLE ROLE OF IL-10 IN TRANSPLANTATION TOLERANCE.' * Item D 613 * --- -/--	1-15
<sup>10</sup> Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "A" document member of the same patent family		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search 04 JUNE 1993		Date of Mailing of this International Search Report 28 -06- 1993
International Searching Authority EUROPEAN PATENT OFFICE		Signature of Authorized Officer RYCKEBOSCH A.O.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	INTERNATIONAL IMMUNOLOGY vol. 4, no. 12, 1992, OXFORD, GB pages 1389 - 1397 M-T. BEJARANO ET AL. 'INTERLEUKIN 10 INHIBITS ALLOGENEIC PROLIFERATIVE AND CYTOTOXIC T CELL RESPONSES GENERATED IN PRIMARY MIXED LYMPHOCYTE CULTURES.' cited in the application see page 1396, right column, line 27 - line 36 ---	1-15
P,X	INTERNATIONAL ARCHIVES OF ALLERGY AND IMMUNOLOGY vol. 99, no. 1, 1992, BASEL, CH pages 8 - 15 H. SPITS ET AL. 'FUNCTIONAL CHARACTERIZATION OF HUMAN IL-10.' see page 10, right column, line 48 - line 50 see page 14, left column, line 2 - line 5 -----	1-15



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 93/01665

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 1-10, 12, 14 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

US 9301665  
SA 71011

04/06/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0405980	02-01-91	AU-B- 635058	11-03-93
		AU-A- 6077090	17-01-91
		CA-A- 2062763	29-12-90
		CN-A- 1051393	15-05-91
		JP-T- 4502560	14-05-92
		WO-A- 9100349	10-01-91
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